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PRINCIPAL INVESTIGATOR: Ginette Serrero, Ph.D.

CONTRACTING ORGANIZATION: University of Maryland
Baltimore, Maryland 21201

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Breast cancer is a major cause of morbidity and mortality in women worldwide. Breast tumors are initially estrogen-dependent but eventually become estrogen-independent and refractory to anti-estrogen therapy. Thus, it is very important to develop new insights into breast cancer. Our approach has been to analyze the cellular changes associated with highly tumorigenic properties. We have characterized a novel growth factor, PC-cell derived growth factor (PCDGF) (epithelin/granulin precursor) in human breast carcinoma cells. This application investigates the role of PCDGF in the proliferation of human breast cancer cells. In estrogen-receptor positive cells, PCDGF expression was stimulated in a dose and time-dependent fashion by estradiol and inhibited by tamoxifen. In estrogen-receptor negative cells, PCDGF was constitutively overexpressed. PCDGF acted as an autocrine growth factor both for ER⁺ and ER⁻ cells. Most importantly, inhibition of PCDGF expression by antisense PCDGF cDNA transfection in ER⁻ breast carcinoma led to a 90% inhibition of tumor incidence and size. These characteristics make PCDGF an important factor involved in the tumorigenicity of breast carcinoma. Future goals are to further investigate PCDGF role on the proliferation of breast cancer cells and examine PCDGF expression in human breast cancer biopsies at different stages of the disease.

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Final Progress Report for Grant application DAMD 17-96-1-6072
Epithelin/granulin precursor in human breast cancer cells
Ginette Serrero, Ph.D. Principal Investigator

INTRODUCTION

The goals of this grant application were to investigate the expression and the role of a growth factor PC-Cell Derived Growth Factor (PCDGF) in human breast cancer cells.

PCDGF is a novel autocrine growth factor, originally purified in the PI's laboratory as a growth factor for the highly tumorigenic teratoma-derived cell line PC. Structural characterization of PCDGF indicated that it was an 88 kDa glycoprotein with a 20 kDa carbohydrate moiety (Zhou et al, 1993). Amino-acid sequencing indicated that PCDGF corresponded to the precursor of a novel family of 6 kDa double cysteine-rich polypeptides, called epithelins or granulins, originally purified from kidney extracts or granulocyte extracts (Plowman et al, 1992; Bandhary et al, 1992).

Comparative studies of PCDGF expression and action indicated that expression of PCDGF was very low in non-tumorigenic cells from which PC cells was derived and increased in tumorigenic derivatives in proportion with their tumorigenicity. Inhibition of PCDGF expression in PC cells by transfection of antisense PCDGF cDNA resulted in a complete inhibition of *in vivo* tumor growth (Zhang and Serrero, 1998). These results suggested that PCDGF overexpression in PC cells was associated with their high tumorigenicity.

To further investigate this possibility, it was demonstrated that: 1) treatment of PC cells with an anti-PCDGF neutralizing antibody inhibited their growth, confirming that PCDGF acted as an autocrine growth factor for the tumorigenic PC cells and 2) inhibition of PCDGF expression in PC cells by transfection of antisense PCDGF cDNA resulted in a complete inhibition of *in vivo* tumor growth (Zhang et al, 1998). These studies indicated that an acquisition of an autocrine production of PCDGF resulted in the deregulated growth of PC cells leading to tumor formation *in vivo* and that PCDGF acted as a tumorigenic growth factor.

These results demonstrated for the first time that the inhibition of PCDGF action or expression constitutes a potent and specific approach to inhibit tumor formation.

Based on these results, screening of human tumors for PCDGF expression was carried out and identified human breast cancer cells as requiring PCDGF for growth and where a correlation between autocrine PCDGF production and tumorigenicity could be demonstrated.

Because of the importance of PCDGF as a tumorigenic factor and since PCDGF was expressed in human breast cancer, the IDEA grant proposed to investigate the biological importance of PCDGF in the growth of breast cancer cells.

RESULTS OBTAINED DURING THE GRANT PERIOD

A) Original Specific aims:

- 1- Comparison of PCDGF expression in normal human mammary epithelial cells and in ER⁺ and ER⁻ breast carcinoma cells.
- 2- Biological activity of PCDGF in mammary epithelial cells and in mammary carcinoma cells
- 3- Effect of inhibition of PCDGF expression (antisense approach) and action (neutralizing antibody or competitive inhibitor approaches) on the growth of malignant breast carcinoma *in vitro* and *in vivo*.

Within the funding period of the grant application all specific aims proposed were fulfilled and even additional results that had not been initially proposed but that were a logical development of the experiments proposed were obtained as summarized below.

B) Accomplishments based on the Specific aims.

1) Overexpression of PCDGF in human breast cancer

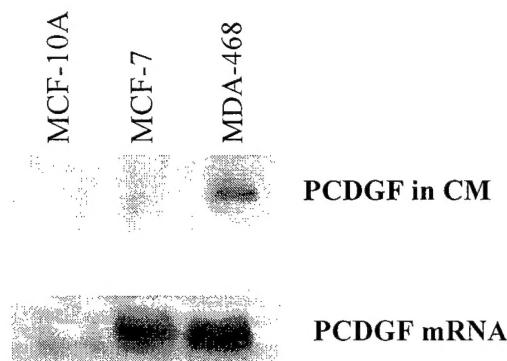


Fig. 1: PCDGF mRNA and protein expression in the human non-tumorigenic MCF-10A and in human breast carcinoma cells MCF-7 (ER+) and MDA-MB-468 (ER-). PCDGF mRNA expression was analyzed by Northern blot analysis (bottom panel). Equal loading of RNA was determined by ethidium bromide staining of samples. Culture medium was examined for the presence of secreted PCDGF protein by immunoprecipitation and western blot analysis (WB, top panel). Samples for WB were normalized to the same cell number.

Screening of human tumors revealed that PCDGF was expressed and secreted by human breast cancer cells. Moreover, determination of the proliferative response of several cell lines to PCDGF indicated that the mouse mammary epithelial cells C57 MG were growth stimulated by PCDGF in a dose-dependent fashion with a maximal stimulation at 150 ng/ml (2 nM). These two observations prompted us to investigate in detail the expression of PCDGF in several human breast cancer cells. PC-cell derived growth factor mRNA and protein expression were first examined in the immortalized non-tumorigenic MCF-10A cells, in the tumorigenic estrogen receptor positive (ER+) MCF-7 cells and estrogen receptor negative (ER-) MDA-MB-468 cells (fig 1). Northern blot and Western blot analysis showed that PCDGF mRNA and protein expression were very low in the MCF-10A cells and dramatically increased in the ER+ MCF-7 cells and the ER- MDA-MB-468 cells. PCDGF expression was also found in T47D another other ER+ cell line. A high level of expression was also found in other ER- cell lines such as MDA-MB-453 and MDA-MB-231.

These results suggest that a high level of PCDGF was found in cells having the highest degree of malignancy.

The results presented above raised two questions: 1) whether PCDGF expression in the ER+ cells is under estradiol control; 2) whether PCDGF stimulates the growth of breast cancer cells in an autocrine fashion. To answer these questions, several experiments were carried out as described below.

2) PCDGF expression is regulated by estradiol in ER+ breast cancer cells (Lu and Serrero, 1999a).



Figure 2: Effect of E2 on PCDGF expression in the ER+ MCF-7 Cells.

Left panel: Time course of the effect of E2 (10^{-9} M) on PCDGF mRNA expression in MCF-7 cells. Right panel: Dose response effect of E2 on PCDGF mRNA expression.

These experiments and other relevant data on E2 effect on PCDGF expression in ER⁺ cells are described in Lu and Serrero (1999a), provided in the Appendix.

Using two different human breast carcinoma ER⁺ cell lines (T47D and MCF-7), it was shown that 17-β estradiol (E2) stimulated PCDGF mRNA and protein expression in a time and dose-dependent fashion (Lu and Serrero, 1999, provided in the Appendix). Maximal stimulation was observed after a 10-hour exposure with 10⁻⁹ M E2. Progesterone had no effect. E2 stimulatory effect of PCDGF expression was blocked by the anti-estrogen tamoxifen and also by actinomycin D suggesting a transcriptional control of PCDGF expression by estrogen. In the estrogen receptor negative (ER⁻) MDA-MB-468 cells, PCDGF expression was constitutively high (Lu and Serrero, 1999a).

3) PCDGF mediates estrogen-dependent growth of human breast cancer cells..

Since E2 stimulated PCDGF expression in ER⁺ cells as well as the proliferation of breast cancer cells, we investigated whether PCDGF could replace E2 to stimulate the proliferation of cells maintained in estrogen-depleted medium. As shown in figure 3, PCDGF stimulated the proliferation of MCF-7 cells maintained in the absence of E2 in a dose-dependent fashion. The maximal stimulation observed with PCDGF (100 ng/ml and above) was similar to that obtained with the optimal concentration of E2 (10⁻⁹M).

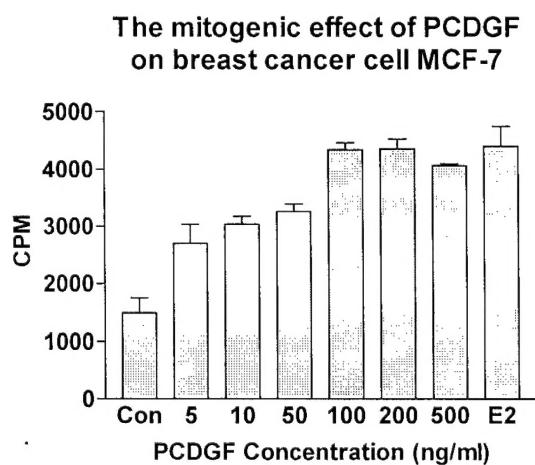


Fig. 3: Effect of human PCDGF on the growth of MCF-7 cells cultivated in the absence of E2.

MCF-7 cells were cultivated in estradiol-depleted α-MEM medium as described in Lu and Serrero (1999b). Proliferation was determined by ³H-thymidine uptake in DNA after 24 hours exposure to PCDGF or E2 and expressed in CPM/well. PCDGF stimulated the proliferation of MCF-7 cells in a dose dependent fashion similarly to E2 (10⁻⁹M). Values are expressed as means ± SD.

Based on these data, it was then hypothesized that PCDGF produced by the breast cancer cells acted as an autocrine growth factor and mediated, at least partially, the stimulatory effect of estradiol.

In order to explore this possibility, we first demonstrated that the growth of MCF-7 cells, cultivated in the presence of E2, when endogenous PCDGF expression is high, was specifically inhibited in a dose dependent fashion by treating the cells with neutralizing anti-human PCDGF polyclonal antibody. Similar doses of non-immune IgG had no effect (fig. 4).

Mitogenic effect of E2 can be neutralized by AntiPCDGF Ab

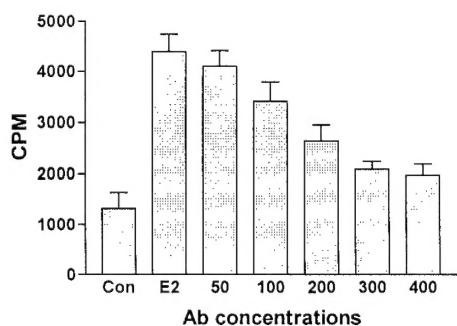


Fig. 4: Effect of anti-PCDGF neutralizing antibody on MCF-7 cell growth.

MCF-7 cells were cultivated in the presence E2 (10⁻⁹M) alone or in the presence of increasing concentrations of affinity purified anti-PCDGF IgG. After 24 hrs, ³H-thymidine was added to the medium to measure DNA synthesis. As a control, non-immune IgG added at similar doses did not affect the growth stimulation of E2. Values are expressed as means ± SD.

Anti-PCDGF neutralizing antibody did not inhibit the proliferative effect of unrelated growth factors, such as IGF-II (data not shown), indicating the specificity of the effect of anti-PCDGF antibody.

4) Constitutive overexpression of PCDGF in MCF-7 cells renders the cells independent from estrogen requirement and leads to tamoxifen resistance

Experiments were then carried out to investigate whether the autocrine production of PCDGF stimulated breast cancer cell growth by mediating some of E2 stimulatory effect. For this purpose, we have isolated MCF-7 cells overexpressing PCDGF in a constitutive fashion by transfecting the cells with PCDGF cDNA ligated into the pcDNA3 expression vector (Lu and Serrero, manuscript in preparation) to determine if these overexpressing cells could by-pass the requirement of E2 to proliferate.

Several clones overexpressing PCDGF were selected and analyzed by measuring PCDGF mRNA expression by Northern blot analysis and PCDGF protein by Western blot analysis of cell lysates and conditioned medium. Empty vector transfected cells were selected as controls. All selected clones (10 total) showed the same properties. The data obtained with one representative clone are shown in Fig. 5. MCF-7 cells overexpressing PCDGF (O4) could proliferate in the absence of E2 in contrast to untransfected or vector control MCF-7 cells that proliferated only when E2 was added to the medium.

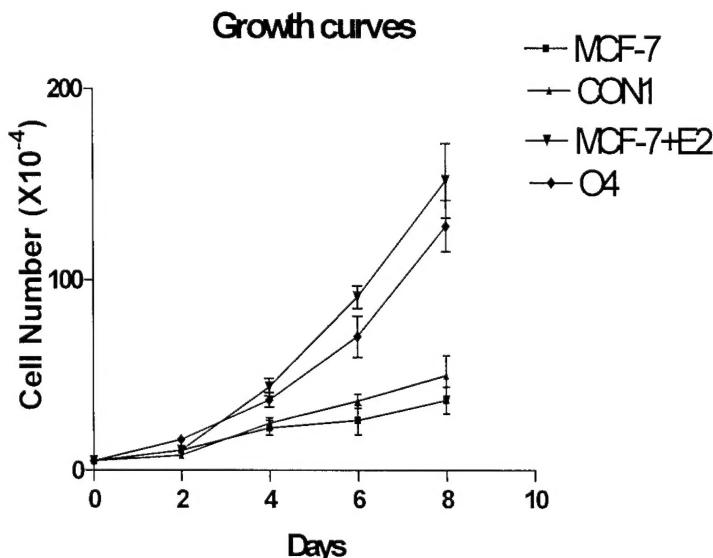


Fig 5: Overexpression of PCDGF in MCF-7 cells stimulates their proliferation in the absence of E2.

MCF-7 cells overexpressing PCDGF by stable transfection of PCDGF cDNA were isolated (O4). Growth curves indicate that O4 cells can proliferate in the absence of E2 whereas empty vector control MCF-7 (CON1) and wild type MCF-7 cells only adequately proliferated when E2 was present in the culture medium. Values are expressed as means \pm SD.

These data indicate that the PCDGF-overexpressing cells had acquired a growth advantage over the vector control MCF-7 cells since O4 cells were able to proliferate even in the presence of estradiol. Measurement of E2 receptor expression in O4 showed no change in expression when compared to control MCF-7 cells. Based on these results, we then investigated the ability of the anti-estrogen tamoxifen to inhibit the growth of control MCF-7 cells and PCDGF- overexpressing O4 cells.

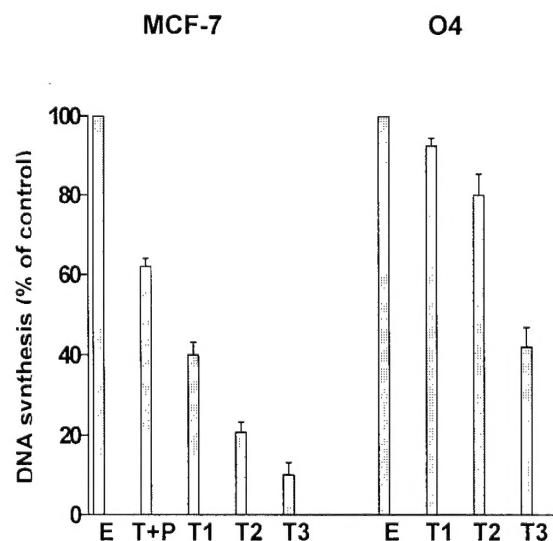


Fig.6: Comparison of MCF-7 and O4 cells response to Tamoxifen.

MCF-7 and O4 cells were plated in 24-well plates at 10⁵ cell / well in 5 % FBS-DME-F12 medium.. 2 days later, the medium was replaced with PFMEM medium followed by serum-free α -MEM medium after 24h. Cells from triplicate wells received 10⁻⁹ M E2 alone (E) or in the presence of Tamoxifen: T1: 0.1 μ M, T2: 0.5 μ M, T3: 1 μ M. 24h later, ³H-thymidine was added. After a 5h labeling, cells were lysed and radioactivity counted. In MCF-7 group, T+P: PCDGF 200 ng/ml and Tamox 1 μ M. Values were calculated as means \pm SD and then expressed as % of controls corresponding to cells not treated with tamoxifen.

In summary, PCDGF is an autocrine growth factor that mediates the growth promoting effect of E2 on breast cancer cells. The PCDGF-overexpressing cells proliferated in the absence of E2 and acquired a growth advantage when compared to control cells. They also displayed resistance to tamoxifen since they were not growth inhibited by tamoxifen doses shown to completely inhibit the growth of control cells (Lu and Serrero, submitted to publication). Addition of 200 ng/ml PCDGF to MCF-7 cells treated with 1 μ M tamoxifen could partially overcome the inhibition caused by tamoxifen.

These results suggest that PCDGF overexpression in breast cancer cells contributes 1) to the transition from estrogen-dependent state to independent state observed in human breast cancer cells and 2) to the acquisition of tamoxifen resistance by PCDGF overexpressing cells.

5) PCDGF activates MAP kinase activity in MCF-7 cells

Studies of PCDGF receptor signaling in MCF-7 cells indicate that PCDGF stimulated the proliferation of breast cancer cells by activating MAP kinase pathway. This was shown by the fact that PCDGF stimulatory effect on MCF-7 cell proliferation was inhibited by treatment of the cells with the MAP kinase inhibitor PD 98059 in a dose dependent fashion. The P-I-3-kinase inhibitor LY294002 had moderate effect (fig 7A).

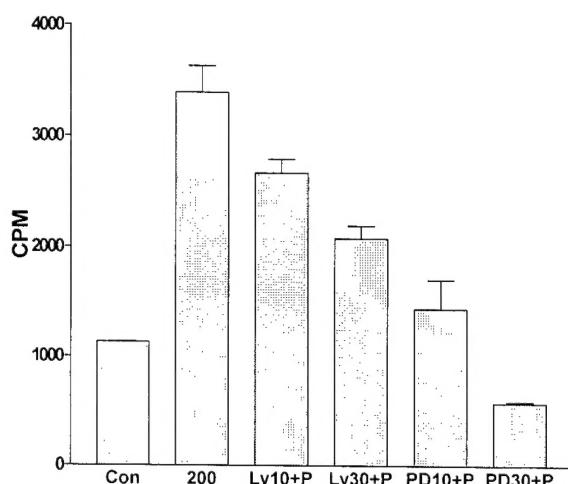


Fig. 7 A: PCDGF effect on DNA synthesis in MCF-7 cells is inhibited by MAP kinase inhibitor PD98059.

MCF-7 cells were cultivated as in fig.6. For treatment with inhibitors, cells were preincubated with LY 294002 or PD098059 for 10 min and 60 min respectively. Cells were then treated for 24 hrs with vehicle only (Con) or with 100 ng /ml PCDGF alone (P) or in the presence of either LY294002 10 μ M (Ly10) or 30 μ M (Ly30) or with PD098059 10 μ M (PD10) or 30 μ M (PD30). 24h later, 3 H-thymidine was added. After a 5h labeling, cells were lysed and radioactivity counted by liquid scintillation counter. Results are expressed as mean \pm SD of triplicate experiments.

We also showed by using in vitro MAP kinase assay that PCDGF stimulated MAP kinase activity in a dose-dependent fashion in MCF-7 cells (fig. 7B). This effect was blocked by PD98059. In O4 cells, basal MAP kinase activity was elevated due to the fact that PCDGF is constitutively produced in these cells.



Fig 7B: PCDGF stimulates MAP kinase activity in MCF-7 cells:

MCF-7 cells were cultured in 10 cm dishes in the same culture conditions as described in fig 7A. For the assay, the cells were treated for 10 min in the conditions described below. Cells were washed twice with ice-cold PBS and lysed on ice for 30 min in lysis buffer in the presence of protease inhibitors followed by centrifugation at 12,000 g for 15 min at 4 °C. Immunoprecipitation of MAP Kinase was performed by incubating equal amount of

supernatant (1mg) with 4 μ g of Erk2 polyclonal antibody for 2h at 4 °C followed by addition of 30 μ l of protein A Sepharose beads. After 1h, beads were washed and the kinase assay was carried out at 30°C for 20 min in the presence of 200 μ g/ml myelin basic protein (MBP) and 10 μ Ci of [γ -ATP]. The samples were analyzed by SDS-PAGE on a 15% polyacrylamide gel followed by autoradiography. Intensity of phosphorylated MBP bands was

quantified by densitometric scanning of the X-ray film. Intensity of the Erk1 and Erk2 signals in the samples, determined by western blot analysis with Erk2 antibody that recognizes both Erk1 and Erk 2, was used as internal controls to normalize MAP Kinase signals. Sample loading sequence: 1, O4 cells; 2-5: MCF-7 cells: 2: untreated control; 3, treated with PCDGF at 50 ng / ml; 4, PCDGF at 200 ng / ml; 5, PCDGF 200 ng / ml and 30 μ M PD098059.

Experiments are currently underway in our laboratory to examine in detail the signaling pathway downstream of MAP kinase activated by PCDGF in MCF-7 cells particularly in comparison with the pathways stimulated by estradiol.

6) Inhibition of PCDGF expression in MDA-MB-468 cells inhibits tumor formation.

Since we have shown that PCDGF was overexpressed in malignant breast carcinoma cells such as the ER⁻ MDA-MB-468 cells, in vivo animal studies were carried out to investigate whether the autocrine production of PCDGF was required for tumor formation. For this purpose, we developed stable cell lines where PCDGF expression had been inhibited by transfection of antisense PCDGF cDNA. Empty vector (pcDNA3) transfected MDA-MB-468 cells were used as controls. Several clones were isolated and growth properties of antisense and control cells were examined. Results obtained with one representative antisense clone AS3 are reported here. These experiments were repeated at least 3 times with AS3 and with 2 other antisense clones.

Comparison of the growth properties of vector control and antisense PCDGF transfected MDA-MB-468 cells (AS-3 in fig 8 and table 1) showed that inhibition of PCDGF expression lead to inhibition of cell proliferation *in vitro* (fig. 8) and tumor growth *in vivo*, measured by injecting 2×10^5 cells into female nude mice (table 1).

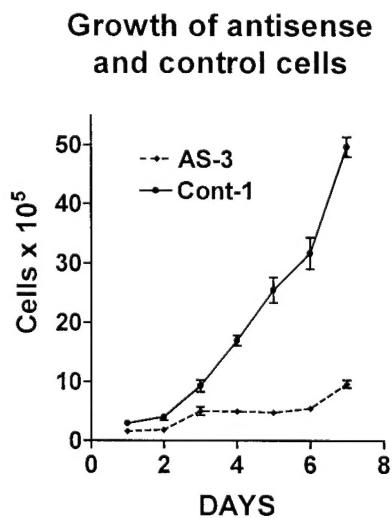


Fig. 8: Growth properties of control empty vector transfected MDA-MB-468 cells and antisense PCDGF cDNA transfected MDA-MB-468 cells.

Cells were cultivated in triplicate in DME/F12 medium supplemented with 10% FBS and counted every day. Antisense transfected cells show a dramatic inhibition of proliferation. This inhibition was reversed by adding exogenous human recombinant PCDGF (data not shown). Data are expressed as means \pm SD.

Table 1: Tumorigenicity of MDA-468 and antisense PCDGF transfected cells

Cells injected	Mice with tumors	Weight of tumors (g)
MDA-MB-468	7/7	0.5 \pm 0.15
AS-3	2/7	0.05 \pm 0.02

Legend of table 1: 6 weeks-old female nude mice were injected subcutaneously with 2×10^5 MDA-MB-468 cells (empty vector transfected control cells) ($n=7$) or 2×10^5 AS-3 cells corresponding to MDA-MB-468 cells stably transfected with pcDNA3 expression vector containing PCDGF cDNA in the antisense orientation. Mice were monitored daily for appearance of tumor. At 45 days, mice bearing tumors were sacrificed and tumor weight determined. Values correspond to mean \pm SD. Data were analyzed by Student's t test and differences between control group and antisense group were considered highly significant ($p<0.001$).

The data of figure 8 and table 1 show that inhibition of PCDGF expression by antisense PCDGF cDNA transfection resulted in a 75 % inhibition of growth both in vitro and in vivo.

These experiments demonstrate for the first time that PCDGF plays an essential role in breast cancer cells tumorigenesis and that inhibition of PCDGF expression led to inhibition of tumor growth.

7) PCDGF is overexpressed in epithelial cells of human ductal carcinoma *

Based on the above in vitro and in vivo studies, we have begun investigating the expression of PCDGF in paraffin-embedded human breast cancer biopsies by immunocytochemistry using an affinity purified anti-human PCDGF antibody. These studies are being performed in collaboration with Dr. Olga Ioffe, Breast Cancer Pathologist at the University of Maryland Cancer Center. The biopsies examined had been classified by cytochemistry, and by examining prognostic markers, such as mitotic index, S phase fractions, and the expression of ER (estrogen receptors) and PR (progesterone receptors).

Preliminary results from the examination of 12 individual biopsies corresponding to ductal and lobular carcinoma indicate a very high PCDGF expression in the epithelial cells in ER⁻/PR⁻ ductal carcinoma grade 3 whereas in normal tissue or benign lobular carcinoma the epithelial cells were negative. Experiments are currently underway in these studies with human breast cancer biopsies to determine whether the changes (temporal and localization) of PCDGF expression in epithelial cells correspond to acquisition of tamoxifen resistance in the patients.

Our results indicate that PCDGF expression in ductal carcinoma correlates with ER⁻/PR⁻ status of the tumors. This suggests that PCDGF overexpression correlates with poor prognosis in these tumors, thereby confirming the importance of PCDGF expression in malignant breast cancer. These data also indicates that PCDGF is a novel biological marker of breast cancer cells.

** Note: These studies were not proposed in the original application and as a result the human subjects category was not checked at the time. However, these studies became a logical extension of our progress. Prior to initiating these experiments, they were submitted to the University of Maryland IRB and were given an exempt status.*

C) Future development based on the results presented above.

Since PCDGF is a novel growth factor, studies were therefore undertaken on the PCDGF receptor.

Characterization of PCDGF receptors (Xia and Serrero, 1998).

We have concentrated our efforts on producing biologically active mouse and human recombinant PCDGF. Since PCDGF is a large glycoprotein, this was achieved either by expressing PCDGF in insect cells (Xia and Serrero, 1998) or in mammalian CHO cells (human PCDGF) (You and Serrero, manuscript in preparation). Binding of ¹²⁵I-labeled PCDGF to a variety of cells was examined using either iodinated PCDGF (fig. 9) Cell lines first investigated included the teratoma 1246, PC cells and mink lung epithelial CCL-64 cells (fig. 9; Xia and Serrero, 1998). Cross-linking of ¹²⁵I-PCDGF to cell surface receptors in all these cell lines indicated the presence of a single 190 kDa cross-linked band corresponding to a 100-105 kDa receptor with 88 kDa recombinant PCDGF. The cross-linked ligand-receptor complex had the same mobility with or without treatment with reducing agent suggesting that PCDGF receptor is a monomeric receptor (Xia and Serrero, 1998). Same results were obtained with MCF-7 cells.

KEY RESEARCH ACCOMPLISHMENTS

- 1- Demonstrated that PCDGF was expressed in human breast cancer cells
- 2- Demonstrated that in estrogen-receptor positive cells, PCDGF expression was stimulated in a dose-dependent fashion by estradiol and inhibited by anti-estrogen tamoxifen whereas in estrogen receptor negative cells, PCDGF expression was constitutively overexpressed.
- 3- Demonstrated that inhibition of PCDGF expression in estrogen receptor negative MDA-MB-468 cells by antisense PCDGF cDNA transfection led to inhibition of tumorigenicity.
- 4- Initiated characterization of PCDGF receptor by binding, Scatchard analysis and affinity cross-linking.
- 5- Initiated studies of PCDGF expression in human breast cancer biopsies

REPORTABLE OUTCOMES

A- Publications, Presentations

- 4 articles published
- 1 manuscript submitted
- 2 manuscripts in preparation
- 3 meeting abstracts for poster presentation at national meetings
- Invited presentation at the 2000 World Congress of tissue culture in a session devoted to growth factors and Cancer. San Diego June 2000. Abstract to be submitted January 31 2000.

B- Degrees obtained that are supported by this award

One graduate student Runqing Lu received his Ph.D. on work supported by this award. Defended September 1999.

C- Funding applied for based on work supported by this award:

Submitted an IDEA grant, NIH RO1, NIH R23 and Susan G. Komen Breast Cancer Foundation grant applications

CONCLUSIONS

• Significance

The data presented above provide compelling evidence that the novel growth factor called PC-Cell Derived Growth Factor (PCDGF) plays a crucial role in the tumorigenesis of human breast cancer. They also provide evidence that overexpression of PCDGF is associated with transition from estrogen dependence to estrogen independence and therefore is crucial for the progression of breast cancer to a refractory state. Examination of human breast cancer biopsies has shown that PCDGF expression in the epithelial compartment of breast lesions is associated with poor prognosis. These results indicate that PCDGF can be considered as a major biological target for the long-term development of approaches to inhibit breast tumor growth.

• Future Perspectives

Based on the results obtained during the funding of this grant application, several important questions are being raised: Several projects are being planned.

1) One is to characterize the receptor for PCDGF. We have developed a purification procedure for the human PCDGF receptor. This project was proposed as an IDEA grant application submitted in 1999.

2) To determine the mechanism underlying overexpression of PCGDF in mammary epithelial cells leading to the development of malignant phenotype in breast cancer cells.

Our long-term goals are to develop therapy and diagnosis screening of breast cancer cells based on inhibiting PCDGF expression and action.

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APPENDIX

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Xia, X and Serrero, G (1998) Identification of cell surface binding sites for PC cell derived growth factor, (epithelin/granulin precursor) on epithelial cells and fibroblasts. Biochem. Biophys. Res. Commun. 245, 539-543.

Zhang, H., and Serrero, G. (1998) Inhibition of tumorigenicity of the teratoma PC cell line by transfection with antisense cDNA for PC cell-derived growth factor (PCDGF). Proc. Natl. Acad. Sci. USA. 95, 14202-14207.

Lu, R and Serrero, G. (1999a) Stimulation of PC cell derived growth factor (epithelin/granulin precursor) expression by estradiol in human breast carcinoma cell line MCF-7. Biochem Biophys Res.Commun. 256, 204-207.

Lu, R and Serrero, G (1999b) Resveratrol a natural product from grape exhibits antiestrogenic activity and inhibits the growth of human breast cancer cells. *J. Cell Physiol* 179, 297-304.

Lu, R and Serrero, G (2000) Inhibition of tumorigenicity of the ER⁺ breast carcinoma MDA-MB-468 cells by transfection with antisense cDNA for PC cell-derived growth factor (PCDGF). Submitted for publication

A copy of each published paper is provided in Appendix with this report. For paper submitted, only the abstract page is provided.

Meeting Abstracts submitted during the course of grant support.

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Epithelin/granulin expression in human breast carcinoma cell lines.

Lu, R and Serrero, G

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Multiple forms of p55PIK, a regulatory subunit of phosphoinositide 3-kinase, are generated by alternative initiation of translation.

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Xianmin Xia, Ph.D. Post-doctoral Fellow

Jun You Ph.D. Post-doctoral Fellow (Dr. You replaced Dr. Xia when his post-doctoral stay was completed)

Runqing Lu: Graduate Student

APPENDIX

Xia, X and Serrero, G (1998) Identification of cell surface binding sites for PC cell derived growth factor, (epithelin/granulin precursor) on epithelial cells and fibroblasts. *Biochem. Biophys. Res. Commun.* 245, 539-543.

Zhang, H., and Serrero, G. (1998) Inhibition of tumorigenicity of the teratoma PC cell line by transfection with antisense cDNA for PC cell-derived growth factor (PCDGF). *Proc. Natl. Acad. Sci. USA.* 95, 14202-14207.

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Lu, R and Serrero, G (2000) Inhibition of tumorigenicity of the ER⁺ breast carcinoma MDA-MB-468 cells by transfection with antisense cDNA for PC cell-derived growth factor (PCDGF). Submitted for publication Abstract only.

Inhibition of tumorigenicity of the teratoma PC cell line by transfection with antisense cDNA for PC cell-derived growth factor (PCDGF, epithelin/granulin precursor)

HAI DI ZHANG* AND GINETTE SERRERO†

Department of Pharmaceutical Sciences, University of Maryland School of Pharmacy and Program of Oncology, Marlene and Stewart Greenebaum Cancer Center of the University of Maryland, 20 North Pine Street, Baltimore, MD 21201-1180

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ABSTRACT The PC cell line is a highly tumorigenic, insulin-independent, teratoma-derived cell line isolated from the nontumorigenic, insulin-dependent 1246 cell line. Studies of the PC cell growth properties have led to the purification of an 88-kDa secreted glycoprotein called PC cell-derived growth factor (PCDGF), which has been shown to stimulate the growth of PC cells as well as 3T3 fibroblasts. Sequencing of PCDGF cDNA demonstrated its identity to the precursor of a family of 6-kDa double-cysteine-rich polypeptides called epithelins or granulins (epithelin/granulin precursor). Since PCDGF was isolated from highly tumorigenic cells, its level of expression was examined in PC cells as well as in nontumorigenic and moderately tumorigenic cells from which PC cells were derived. Northern blot and Western blot analyses indicate that the levels of PCDGF mRNA and protein were very low in the nontumorigenic cells and increased in tumorigenic cell lines in a positive correlation with their tumorigenic properties. Experiments were performed to determine whether the autocrine production of PCDGF was involved in the tumorigenicity of PC cells. For this purpose, we examined the *in vivo* growth properties in syngeneic C3H mice of PC cells where PCDGF expression had been inhibited by transfection of antisense PCDGF cDNA. The results show that inhibition of PCDGF expression resulted in a dramatic inhibition of tumorigenicity of the transfected cells when compared with empty-vector control cells. These data demonstrate the importance in tumor formation of overexpression of the novel growth factor PCDGF.

The C3H mouse teratoma-derived cell line 1246 is an adipogenic cell line requiring insulin to proliferate and differentiate in defined medium (1, 2). Insulin-independent variant cell lines were isolated from 1246 cells maintained in insulin-free medium. One cell line called 1246-3A was analyzed in detail and shown to have lost the ability to differentiate and to have become moderately tumorigenic when 10^6 cells were injected into syngeneic host C3H mice (3). It was shown that the 1246-3A cells synthesized and secreted several factors that affected their proliferation and differentiation and the factors were biochemically characterized and identified (4–6). To establish a cell culture model of increased tumorigenicity, an *in vitro*–*in vivo* shuttle technique (7) was applied to isolate from the 1246-3A cells a highly tumorigenic cell line called PC (5). These highly tumorigenic PC cells gave rise to tumors when 10^4 cells were injected s.c. into syngeneic C3H mice (5). These three cell lines ranging from differentiating and nontumorigenic cells to differentiation-deficient, highly tumorigenic cells represented a unique model system to analyze the cellular and

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biochemical changes associated with the acquisition of tumorigenic properties. Comparative studies of the growth properties of the cell lines indicated that PC cells had lost the ability to respond to the growth factors required by 1246 and 1246-3A cells and instead became dependent on their own conditioned medium for proliferation (8). The factor responsible for this growth stimulation, PC cell-derived growth factor (PCDGF), was purified to homogeneity and sequenced (9). PCDGF was an 88-kDa glycoprotein consisting of a 68-kDa core polypeptide and a 20-kDa carbohydrate moiety (8), shown by amino acid and cDNA sequencing to be identical to the epithelin/granulin precursor (9, 10). Epithelin and granulins are 6-kDa double-cysteine-rich polypeptides originally purified from rat kidney (11) and from human granulocyte extracts (12). Although no function was attributed to granulins during their purification and characterization, epithelins were shown to be dual growth effectors for epithelial cells (10, 11). Cloning and sequencing of epithelin and granulin cDNAs showed that the 6-kDa polypeptides were encoded by a common precursor cDNA (9, 10). This epithelin/granulin precursor corresponded to a 63-kDa polypeptide contained 7.5 repeats of the 6-kDa epithelin or granulin polypeptides and several putative glycosylation sites (9, 10). As PC was a highly tumorigenic cell line that synthesized and secreted PCDGF, it was important to investigate the possible role of PCDGF on the growth of the teratoma cells. For this purpose, two experimental approaches were taken. One was to determine whether PCDGF was expressed preferentially in the highly tumorigenic cells. This was done by comparing the levels of expression of PCDGF mRNA and protein in the tumorigenic PC cell derivatives with the ones found in the nontumorigenic 1246 and moderately tumorigenic 1246-3A parent cells. The second approach was to examine the role of PCDGF expression on the tumorigenic properties of the PC cells by preventing the factor from being synthesized and examining the resulting changes in the growth properties of the cells. This was achieved by transfecting PC cells with an antisense PCDGF cDNA construct that blocked the growth factor expression. This approach has been used widely to examine the effect of inhibiting the expression of growth factors or their receptors on the growth properties of producer cells (13–19). Experiments presented here examined the *in vitro* and *in vivo* growth properties of PC cells in which PCDGF expression had been inhibited by transfection with antisense PCDGF cDNA.

MATERIALS AND METHODS

Cell Culture. Stock cultures of PC and 1246-3A cells were maintained in defined media as described previously (6). 1246

Abbreviations: FBS, fetal bovine serum; PCDGF, PC cell-derived growth factor; CMV, cytomegalovirus.

*Present address: Rheumatology Division, University of Pennsylvania School of Medicine, Philadelphia, PA 19104.

†To whom reprint requests should be addressed. e-mail: gserro@pharmacy.ab.umd.edu.

stock cells were cultivated in DME/F12 nutrient medium (1:1 mixture) (GIBCO BRL) supplemented with 10% fetal bovine serum (FBS) (Gibco). For comparative studies, the three cell lines were cultivated in DME/F12 medium supplemented with 2% FBS.

Northern Blot Analysis of PCDGF mRNA Expression. Total cellular RNA was isolated by RNAzol method (Cinna/Biotex Laboratories, Friendswood, TX). Fifteen micrograms of total RNA was separated by electrophoresis on a denaturing 1.2% agarose gel containing 0.22 M formaldehyde in 1× MOPS (20). RNA samples were blotted onto nitrocellulose membrane (MSSI, Westboro, MA) by overnight capillary transfer in 10× SSC, and then hybridized at 42°C overnight in hybridization solution [50% formamide, 5× SSPE, 1% SDS, 5× Denhardt's solution, 1 μg/ml poly(A), 100 μg/ml denatured salmon sperm DNA] with approximately 10⁶ cpm/ml of randomly primed ³²P-labeled mouse PCDGF cDNA probe to measure PCDGF mRNA expression. Filters were washed and exposed to x-ray film (Kodak X-Omat AR) for autoradiography. Ribosomal protein L32 mRNA (RPL32) was detected as an internal standard for normalizing RNA loading (21).

Immunoprecipitation and Western Blot Analysis of PCDGF Protein. Since PCDGF is a secreted protein, its expression was measured in cell lysates and conditioned media collected in the presence of a protease inhibitor mixture of 200 μM phenylmethylsulfonyl fluoride (PMSF), 1 μM leupeptin/0.5 μM aprotinin, 1 mM EDTA (all obtained from Sigma). Cells were lysed in PBS containing 1% Triton X-100 followed by sonication and centrifugation. For comparative studies of PCDGF expression, the samples used for immunoprecipitation and Western blot analysis were normalized to equivalent cell number (see figure legends), determined by counting cells from duplicate sets of dishes. Immunoprecipitation of PCDGF was carried out by incubating samples for 4 hr with 5 μg of affinity-purified anti-PCDGF IgG conjugated to agarose beads followed by centrifugation at 10,000 × g for 10 min. Immune complexes were resuspended in Laemmli sample buffer (22), boiled for 5 min, and separated by electrophoresis on a 10% polyacrylamide gel in the presence of SDS. Proteins were electrophoretically transferred to nitrocellulose membrane (Schleicher & Schuell). The membranes were blocked with 5% nonfat milk overnight at 4°C and then incubated for 1 hr at room temperature with 10 ng/ml of anti-PCDGF IgG conjugated to horseradish peroxidase, in the presence of 1% BSA. Immunoreactivity was visualized by the enhanced chemiluminescence detection system (Amersham).

Construction and Transfection of Antisense PCDGF cDNA Expression Vector in PC Cells. PC cells were transfected with a PCDGF antisense cDNA fragment cloned into the expression vector pCMV4 (23) as described below. A 228-bp PCDGF cDNA fragment that included the start codon region was obtained by digesting full-length PCDGF cDNA with *Sma*I and *Xba*I enzymes. This fragment was cloned in the antisense orientation into *Xba*I and *Sma*I site of pCMV4, and is referred to hereafter as pAS-PCDGF. Transfection of PC cells with pAS-PCDGF by the calcium phosphate method (20) was performed when the cells reached 80% confluence in DME medium supplemented with 10% FBS. A calcium phosphate precipitate added to the cells contained 20 μg of pAS-PCDGF plasmid, 2 μg of pRSVneo plasmid carrying the neomycin-resistant gene as a selectable marker, and 20 μg of pSK plasmid as carrier. After 7 hr, the cells were shocked with 10% DMSO for 2–3 min, washed twice, and fed with DME/F12 medium supplemented with 10% FBS. One day after transfection, cells were subcultured at a 1:3 ratio and cultivated in serum-supplemented medium in the presence of 400 μg/ml of Geneticin (G-418 Sulfate, GIBCO BRL). This medium first was changed 2 days later and then every 3–4 days thereafter. After 10–14 days, colonies of G418-resistant cells were picked with cloning rings and cultivated in DME/F12 medium sup-

plemented with 10% FBS and 400 μg/ml G418. Control PC cells were transfected with pCMV4 and pRSVneo plasmid DNAs and isolated as described above. The presence of the transfected DNA (pAS-PCDGF or pCMV4) was determined by PCR analysis of genomic DNA isolated from G418-selected transfecants. For empty-vector control cells, PCR analysis was performed by using the primer pair of SP647, 5'-CCTACTT-GGCAGTACATCTACGTA-3', and AP912, 5'-CTGACGGTTCACAAACGAGCTC-3', corresponding to the cytomegalovirus (CMV) promoter region. The sense primer SP647 (described above) and antisense primer SP7 5'-CGAGAATTCAAGGCAGACCATGTGGGT-3' located in the start codon region of PCDGF cDNA were used to test for the presence of antisense PCDGF cDNA in the genomic DNA of antisense transfecants. The transfecants were lysed in buffer A (100 mM KCl/10 mM Tris-HCl, pH 8.3/0.45% Tween 20/0.45% Nonidet P-40) and 120 μg/ml proteinase K (Boehringer Mannheim). Then, they were incubated at 60°C for 1 hr, followed by boiling for 15 min. DNA of each clone (50–100 ng) was used as template for PCRs. DNA from nontransfected cells was used as negative control. Plasmid DNA was used as a positive control. PCR was performed in a 20-μl reaction mixture containing 10 mM Tris-HCl, pH 9.0/50 mM KCl/1.5 mM MgCl₂/0.1% Triton X-100/0.2 mM dNTP/0.5 units *Taq* DNA polymerase (Promega)/20 ng of each primer/50 ng genomic DNA template. The reaction tubes were heated to 95°C for 3 min and then subjected to 40 cycles of 95°C for 1 min, 55°C for 2 min, and 72°C for 3 min with a 10-min 72°C extension in a thermocycler (MJ Research, Cambridge, MA). Ten microliters of PCR product was analyzed on a 1% agarose gel and stained with ethidium bromide.

Measurement of Cell Proliferation. Proliferation of pAS-PCDGF and control transfected PC cells was examined. Cells were plated in 12-well plates (Corning) at a density of 3 × 10⁴ cells per well in 2 ml of either 2F defined medium consisting of DME/F12 medium supplemented with 2 μg/ml human fibronectin (Upstate Biotechnology, Lake Placid, NY) and 10 μg/ml human transferrin (Sigma) or DME/F12 medium supplemented with 2% FBS. On day 5, cells were washed with PBS and enumerated with a Coulter counter after trypsinization of cells from duplicate wells.

Tumorigenicity Study. Six-week-old female C3H mice from Taconic Farms (Germantown, NY) were injected s.c. with 10⁶ pAS-PCDGF or control transfected PC cells. The appearance and size of tumors were examined daily. The mice were sacrificed 40 days after injection to measure tumor weight.

All experiments described here were repeated at least twice.

RESULTS

Comparison of PCDGF mRNA Expression in 1246, 1246-3A, and PC Cells. The cell lines 1246 (nontumorigenic), 1246-3A (moderately tumorigenic), and PC (highly tumorigenic) were cultivated in DME/F12 medium supplemented with 2% FBS. When the cells were 80% confluent, total RNA was extracted and PCDGF mRNA expression was measured by Northern blot analysis using a radiolabeled PCDGF cDNA probe. RPL32 mRNA expression was measured as an internal control for equal RNA loading. As shown in Fig. 1, the level of expression of PCDGF was very low in the nontumorigenic 1246 cells and in the moderately tumorigenic 1246-3A cells. In contrast, PCDGF mRNA expression increased by at least 20-fold in the highly tumorigenic PC cells. The size of the PCDGF mRNA transcript expressed by the cells was 2.2 kb.

PCDGF Protein Expression in the Three Cell Lines. Next we examined the expression of PCDGF protein in the three cell lines. Since PCDGF is a secreted protein (8), we examined its level not only in cell lysates but also in the culture medium of the three cell lines by a combination of immunoprecipitation and Western blot analysis by using an anti-PCDGF antibody.

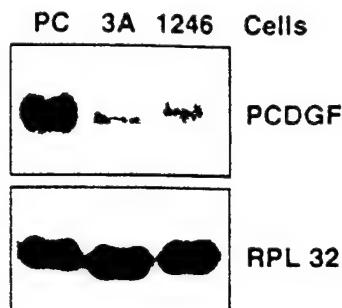


FIG. 1. Comparison of PCDGF mRNA expression in 1246, 1246-3A, and PC cells. Cells were cultured in DME/F12 medium supplemented with 2% FBS until 80% confluent. Medium was changed 24 hr before RNA was collected. Fifteen micrograms of total RNA was analyzed by Northern blot with 32 P-labeled PCDGF cDNA probe to measure PCDGF mRNA expression. Ribosomal protein RPL32 mRNA expression was used as internal standard for RNA loading.

As shown in Fig. 2, the level of expression of PCDGF in cell lysates and conditioned media of the highly tumorigenic PC cells was much higher than in 1246 and 1246-3A cells, similar to the differences observed for PCDGF mRNA expression (Fig. 1). PCDGF protein expression was very low in the 1246-3A cell lysate and culture medium and undetectable in 1246 cells. The data presented in Figs. 1 and 2 suggest that the level of PCDGF expression in the three cell lines correlated with the degree of tumorigenicity of the cells. Based on these results, it was hypothesized that PCDGF overexpression in PC cells was directly associated with their tumorigenic properties. To examine this hypothesis, experiments were carried out to isolate PC cells in which PCDGF expression had been inhibited by stable transfection with PCDGF antisense cDNA and to compare the tumorigenic properties of these antisense transfected cells with empty-vector transfected PC cells.

Isolation of Antisense PCDGF Transfected PC Cells. PC cells were cotransfected with the pAS-PCDGF plasmid (Fig. 3) and with the pRSVneo plasmid as described under *Material and Methods*. Control PC cells were cotransfected with empty pCMV4 and pRSVneo plasmids. In both cases, transfected cells were selected for growth in medium containing G418. Colonies were assayed for the presence of the transfected plasmids by PCR analysis of genomic DNA (Fig. 4). Antisense pAS-PCDGF and empty-vector control transfected cells were tested further to determine the level of PCDGF protein expression by Western blot analysis (Fig. 5). All antisense clones tested exhibited a significantly reduced level of PCDGF expression both in cell lysates and in conditioned media when

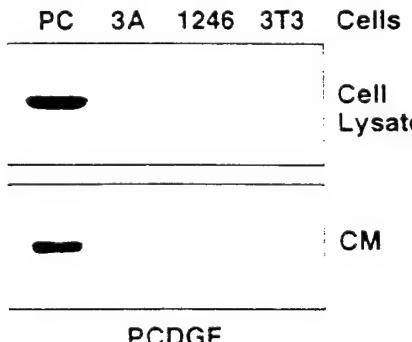


FIG. 2. PCDGF protein expression in 1246, 1246-3A, and PC cells. Cells were cultivated as described in the legend of Fig. 1. Cell lysates and conditioned media were collected and normalized by cell number. Samples corresponding to 18×10^6 cells (cell lysates) and 3×10^6 cells (conditioned media) were used to measure PCDGF expression by immunoprecipitation and Western blot by using an anti-PCDGF antibody as described under *Materials and Methods*.

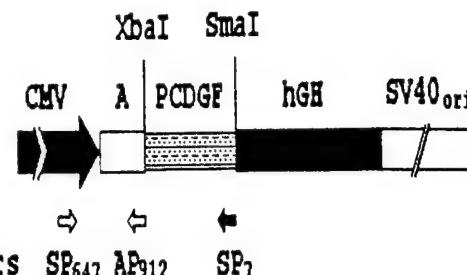


FIG. 3. Construction of antisense PCDGF cDNA. A 228-bp PCDGF cDNA fragment (-45 to +183 bp) including the start codon region was ligated in the antisense orientation into the *Xba*I-*Sma*I site of pCMV4 expression vector. Immediate early promoter region (CMV, stippled block), a DNA copy of a segment of the alfalfa mosaic virus 4 RNA that contains a translational enhancer (A), transcription termination and polyadenylation signals from the human growth hormone gene (hGH, gray block), and the simian virus 40 (SV40) origin of DNA replication and early region enhancer sequences (SV40_{ori}, white block) are shown. Locations of primers used for PCR analysis of transfectants are indicated.

compared with empty-vector controls (P6 and P14) and with wild-type PC cells. Three antisense clones (AS-II1, AS-II15, and AS-II18) and two empty-vector control clones (P6 and P14) were chosen and studied further. Inhibition of PCDGF protein expression was observed in the three antisense transfected clones to varying degrees (Fig. 5). The level of PCDGF protein secreted in the conditioned media of empty-vector control cells (P14 and P6) was similar to that secreted by untransfected PC cells.

In Vitro Growth Properties of Antisense and Control Transfected Cells. The morphology of antisense and control transfected cells was examined. Phase-contrast micrographs indicated that the pAS-PCDGF transfected cells did not spread as well as the control cells and they maintained a rounded morphology when compared with control transfected PC cells (Fig. 6).

Growth of both types of transfected cells was measured in serum-supplemented medium and in serum-free defined medium (Fig. 7). pAS-PCDGF transfected cells cultivated either in defined medium (Fig. 7 Lower, 2F medium) or in serum-containing medium (Fig. 7 Upper) had a reduced proliferative capacity when compared with control transfected cells and

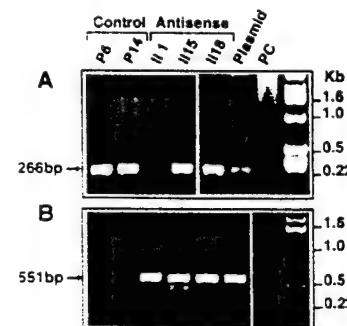
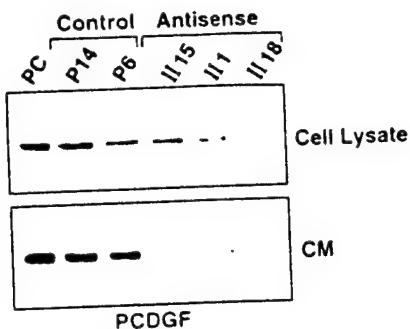


FIG. 4. PCR analysis of antisense and control transfected clones. PCR analysis of DNA from the selected clones was performed with primer pairs as described in *Materials and Methods*. (A) SP647 (sense primer) and the AP912 (antisense primer), both located in the CMV promoter (Fig. 3), were used to amplify a 266-bp fragment from cells transfected with the CMV promoter expression vector (antisense and empty vector transfected cells). (B) Sense primer SP647 (described above) and antisense primer SP7, located in the start codon region of PCDGF cDNA (Fig. 3), were used to amplify a 551-bp DNA fragment in the pAS-PCDGF transfectants only. No amplified band was obtained with DNA from control transfected cells. pAS-PCDGF plasmid DNA and genomic DNA from nontransfected PC cells were used as positive and negative control template for PCR, respectively.



5. PCDGF protein expression in antisense and control transfectants. PC cells and antisense and control transfectants were plated in DME/F12 medium supplemented with 2% FBS. The medium was replaced with fresh medium 24 hr before the samples were taken. Cell lysates and conditioned media were normalized by cell numbers of 18×10^5 and 3×10^5 cells, respectively, and were immunoprecipitated with anti-PCDGF IgG and analyzed by Western blot as described in *Materials and Methods*.

transfected PC cells cultivated in similar culture conditions. After 5 days in culture, the number of pAS-PCDGF transfected cells was reduced by 90% in defined medium and by 70% in serum-supplemented medium compared with control cells cultivated in the same conditions. Moreover, after 5 days, the number of cells in defined medium was 10% of those in serum-containing medium. In contrast, empty-vector control cells had the same proliferation capacity as nontransfected PC cells, and they displayed only a 50% reduction in cell number after 5 days in defined medium when compared with serum-containing medium.

Tumorigenic Properties of Antisense and Control PC Cells. The tumorigenic properties of the three pAS-PCDGF transfected clones and two empty-vector control clones were examined by s.c. injection of 10^6 cells per mouse into 6-week-old syngeneic C3H female mice. Tumor formation was followed by daily monitoring of the mice, and tumor growth was determined by measuring the dimensions of the tumors. Fig. 8 shows mice injected with control cells and pAS-PCDGF transfected cells 40 days after injection. The mice were sacrificed and the tumors were collected and weighed. As shown in Table 1, all the mice injected with either one of the control transfected PC cell lines (P14 and P6) developed tumors rapidly. Tumors were visible as early as 15 days after injection for both the control transfectants and nontransfected PC cells. These re-

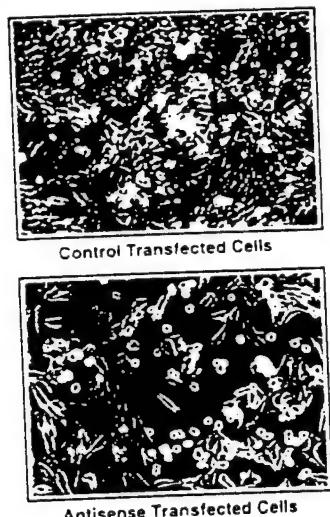
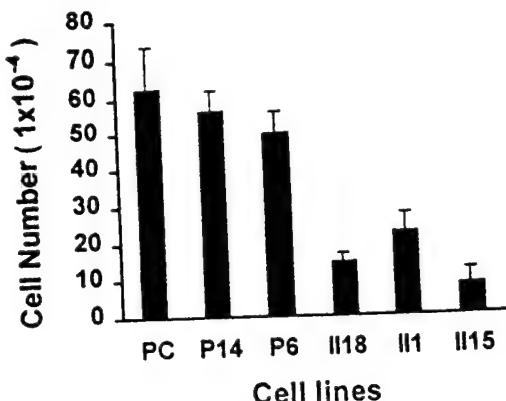


Fig. 6. Morphology of antisense and control transfectants in monolayer culture. Phase-contrast micrographs of antisense and control transfectant cells. ($\times 100$.)



Cell Growth in 2F

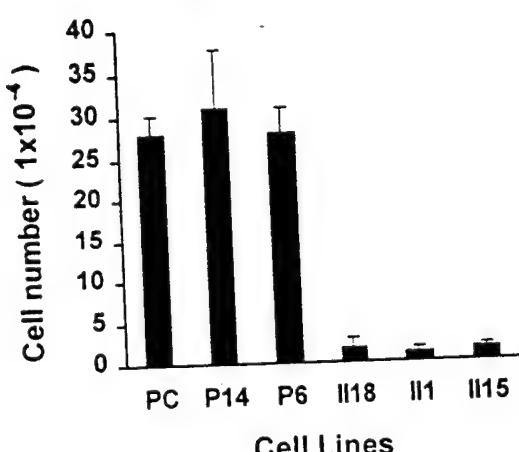


Fig. 7. *In vitro* growth properties of antisense and control transfected clones. PC cells, antisense cDNA transfected cells, and empty vector control transfected cells were plated either in DME/F-12 medium supplemented with 2 μ g/ml human fibronectin and 10 μ g/ml human transferrin (2F medium, *Lower*) or with 2% FBS added 12 hr after plating (*Upper*). At day 5, cells from duplicate wells were trypsinized and counted with a Coulter counter. The experiment was repeated twice. Each bar represents the mean number of cells (\pm SD) calculated from both experiments.

sults indicated that the growth properties of the control cells had not been affected significantly by the transfection and selection processes. In contrast, all three clones that had been transfected with pAS-PCDGF showed a marked inhibition of tumor growth. For AS-II1 and AS-II18, inhibition was complete because after 40 days, none of the mice injected with either of these clones had tumors. For AS-II15, two of the five mice injected developed tumors, but the inhibition of tumor growth was still significant as AS-II15 tumors were 5–10% of the weight of the control transfected PC cell tumors. Even 60 days after injection in C3H mice, AS-II1 and AS-II18 did not form palpable tumors (data not shown).

DISCUSSION

PCDGF is a growth factor belonging to a family of double-cysteine-rich polypeptides that includes the 6-kDa cysteine-rich polypeptides epithelins and granulins (8–10). PCDGF is secreted as an 88-kDa glycoprotein by the highly tumorigenic PC cell line. PC cells are insulin-independent teratoma cells isolated for their high tumorigenic properties in syngeneic C3H mice (3). Histological analysis of the tumors generated by

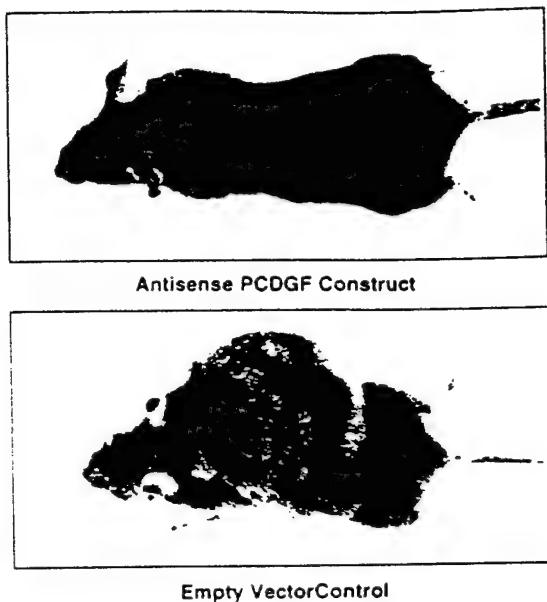


FIG. 8. Comparison of mice injected with antisense and control transfected cells. C3H female mice were photographed 40 days after s.c. injection of 10^6 cells from antisense (Upper) or control PC transfectants (Lower).

s.c. injection of PC cells in C3H mice indicate that PC cells form malignant fibrous histiocytomas (unpublished data). Sequencing of full-length PCDGF cDNA isolated from PC cell cDNA library indicated that PCDGF was identical to mouse epithelin/granulin precursor (10–11) and that PCDGF expressed by the highly tumorigenic cells did not contain any mutations (unpublished data). Time course studies of PCDGF synthesis and secretion in PC cells have shown that PCDGF is secreted as early as 2 hr after synthesis (data not shown). This observation and the fact that PC cells were growth-stimulated by PCDGF (8) and presented PCDGF cell-surface binding sites (24) indicated that secreted PCDGF was an autocrine growth factor for the highly tumorigenic cells. Based on these results, it was hypothesized that PCDGF expression may be increased in tumorigenic cells when compared with their normal counterparts. Increased expression of several growth factors in many different tumor cells, such as IGF-I in glioblastoma (14) and transforming growth factor- α in human breast cancer cells (25), has been observed. The model system consisting of nontumorigenic 1246 cells (1) and moderately tumorigenic 1246-3A (2) and highly tumorigenic PC cells (5) provided an experimental system to investigate directly the role of PCDGF (epithelin/granulin precursor) in tumorigenic cell lines derived from the same nontumorigenic and hormone-responsive parent cell line. The analysis of PCDGF mRNA and

Table 1. *In vivo* tumorigenicity of PC cells transfected with antisense PCDGF cDNA and with empty vector

Cells injected	Day of appearance \pm SD	Mice with tumors	Weight, g, \pm SD
AS-II1	—	0/5	—
AS-II18	—	0/5	—
AS-II15	30 \pm 14	2/5	0.35 \pm 0.05
P14	15 \pm 3	5/5	5.4 \pm 2.0
P6	15 \pm 0	5/5	3.6 \pm 1.9
PC	15 \pm 4	5/5	6.4 \pm 2.6

C3H female mice were injected s.c. with 10^6 cells from PCDGF antisense cDNA transfectants (AS-II1, AS-II18, and AS-II15), control transfectants (P14, P6), or nontransfected PC cells. Average day of tumor appearance, number of mice (of five) with tumors at 40 days, and tumor weight in g \pm SD are provided.

protein levels in the three cell lines indicated that PCDGF expression was undetectable in the nontumorigenic 1246 cells and significantly increased in the highly tumorigenic cells in correlation with the degree of tumorigenicity of the teratoma cells.

To determine whether increased PCDGF expression in PC cells contributed to their high tumorigenic properties, we used the antisense cDNA transfection approach to block PCDGF expression in PC cells and examine their growth characteristics. Antagonizing mRNA with the help of "hybridization competitor" in inhibiting protein synthesis was first introduced by Zamecnik *et al.* (26) and Plesner *et al.* (27). Recent years have seen considerable progress in studying the role of antisense RNA as an inhibitor of oncogenic protein production (13). The development of stable transfected clones with antisense cDNA is advantageous in that it allows a continuous supply of antisense RNA to disrupt protein synthesis and it is well suited for *in vivo* tumorigenic assays. Our results demonstrate that decreasing PCDGF protein synthesis and secretion by expression of antisense PCDGF mRNA in the highly tumorigenic PC cells reduced their growth ability *in vitro* and inhibited their tumorigenicity *in vivo*. Phase-contrast microscopic analysis showed that the antisense transfected cells with inhibited PCDGF expression had a decreased ability to spread on the tissue culture substrate. Many cells were rounded and floated in the culture medium. In contrast, empty vector control transfectants expressing normal PCDGF levels were well spread and grew as well as untransfected PC cells. Localization of PCDGF in PC cells by immunofluorescence with anti-PCDGF antibody revealed PCDGF staining at sites of cell-cell contact (unpublished data). This evidence suggests that PCDGF may affect cell growth by a complex mechanism including autocrine stimulation of PC cell growth and contribution in the regulation of adhesion and communication between cells.

In vivo studies indicated that inhibiting PCDGF expression in the teratoma cells resulted in an inhibition of tumor growth. Comparison of tumorigenicity (Table 1) and Western blot analysis showing the level of residual PCDGF expression in the pAS-PCDGF transfectants (Fig. 5) suggests that the degree of inhibition of tumorigenicity correlates with the degree of inhibition of PCDGF expression in the cells. The two cell lines (AS-II1 and AS-II18) with the lowest level of PCDGF production and secretion were not tumorigenic since none of the injected mice developed tumors even after 60 days while tumors appeared in 15 days in mice injected with control cells. In contrast, AS-II15 cells, in which inhibition of PCDGF expression by antisense PCDGF cDNA transfection was not as efficient, maintained some degree of tumorigenicity (although a reduced one) since two of five mice developed smaller tumors, which first appeared after 36 days. Teratocarcinoma stem cells are known to originate from a breakdown in the normal regulatory processes controlling proliferation and differentiation rather than from mutations in genes responsible for the normal control of cell behavior (28). The abnormal behavior of such embryonic stem cells can be reversed if they are placed in a normal environment. Little is known about the molecular mechanism of the malignant transformation of teratoma. The fact that malignant teratoma arise from relatively undifferentiated cells implies the existence of a relationship between tumorigenesis and differentiation (29). The three cell lines, 1246, 1246-3A, and PC, with increasing tumorigenic properties were derived from a C3H mouse teratoma and provide a good model system to study the molecular mechanisms involved in the loss of differentiation properties and the acquisition of tumorigenic properties. The studies with this model system described here demonstrate that the epithelin/granulin precursor also known as PCDGF is an essential autocrine modulator resulting in the autonomous growth of undifferentiated, highly tumorigenic PC cells *in vitro* and *in*

vivo. The data presented here also demonstrate that overexpression of epithelin/granulin precursor, PCDGF, may play an important function in teratocarcinogenesis and in tumorigenesis.

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Identification of Cell Surface Binding Sites for PC-Cell-Derived Growth Factor, PCDGF, (Epithelin/Granulin Precursor) on Epithelial Cells and Fibroblasts¹

Xianmin Xia* and Ginette Serrero*†,2

*Department of Pharmaceutical Sciences, University of Maryland School of Pharmacy, and †Program of Oncology at the Marlene and Stewart Greenebaum Cancer Center, University of Maryland, 20 North Pine Street, Baltimore, Maryland 21201-1180

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PC cell derived growth factor (PCDGF) is an 88-kDa glycoprotein purified from the culture medium of the highly tumorigenic mouse teratoma-derived cell line PC. PCDGF was shown to stimulate the proliferation of 3T3 fibroblasts and PC cells. Amino acid sequencing of PCDGF indicated its identity to the precursor for the 6-kDa polypeptides epithelins and granulins. In this paper, we investigated the binding of PCDGF to the mink lung epithelial cell line CCL64. Scatchard analysis indicates that ^{125}I -PCDGF binding to CCL64 cells is curvilinear, corresponding to the existence of two classes of binding sites: high affinity binding sites (560 ± 170 sites/cell) with a K_{d1} of 43 ± 15 pM and low affinity binding sites ($16,350 \pm 5900$ sites/cell) with a K_{d2} of 3.9 ± 1.9 nM. ^{125}I -PCDGF was chemically crosslinked to cell surface receptors on CCL64 cells with disuccinimidyl suberate. A major crosslinked band of about 190 kDa with radiolabeled PCDGF was detected after SDS-PAGE, suggesting the presence of PCDGF binding sites with molecular weight of about 120 kDa. ^{125}I -PCDGF crosslinking studies indicate the presence of PCDGF binding sites with a molecular weight similar to those of binding sites on CCL64 cells on the surface of two other PCDGF-responsive cell lines, 3T3 fibroblasts and PC cells. These data suggest that the receptors for PCDGF are widely distributed on cells of distinct embryonic origin.

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The C3H mouse teratoma-derived cell line 1246 is an adipogenic cell line which requires insulin to proliferate and differentiate in defined medium (1, 2). Insulin-independent variant cell lines have been isolated from 1246 cells maintained in the absence of insulin. One of them called 1246-3A cell line was particularly studied (2). These cells are unable to differentiate and become tumorigenic when 10^6 cells are injected into syngeneic hosts C3H mice. By an *in vitro-in vivo* shuttle technique, a highly tumorigenic cell line called PC was isolated. PC cells give rise to tumors even when 10^4 cells are injected into C3H mice (3). Comparison of the growth properties of 1246, 1246-3A and PC cells indicated that PC cells had lost binding and response to several growth factors to which the 1246 and 1246-3A cells responded (3-5). In contrast, PC cells became dependent for their growth at low cell density on the presence of their own conditioned medium. A growth-promoting factor called PC-cell derived growth factor (PCDGF) present in the PC conditioned medium was purified to homogeneity as an 88-kDa glycoprotein (6). Deglycosylation of the protein indicated that PCDGF has a 68-kDa protein core and a 20-kDa carbohydrate moiety (6). N-terminal sequencing and partial sequencing of internal peptide fragments demonstrated that PCDGF contained regions of sequence identity to that deduced from the granulin or epithelin precursor cDNAs (6-8). Definite identification of PCDGF to epithelin/granulin precursor was demonstrated upon cloning full length PCDGF cDNA from a PC cell cDNA library.³ Granulins and epithelins are 6-kDa cysteine-rich polypeptides originally purified from granulocytes (9) and kidney extracts (10). 5 granulins were purified with no known function (9). Epithelins 1 and 2, the two 6-kDa polypeptides purified from rat kidney extracts, were shown to inhibit the growth of A431 cells and the

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* To whom correspondence should be addressed. Fax: 1 410 7060346. E-mail: gserrero@pharmacy.ab.umd.edu.

Abbreviations used: BSA, bovine serum albumin; DME, Dulbecco's modified Eagle's medium; DSS, disuccinimidyl suberate; EGF, epidermal growth factor; FBS, fetal bovine serum; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PCDGF, PC cell-derived growth factor.

³ H. Zhang and G. Serrero, manuscript submitted for publication.

proliferation of the human breast carcinoma cell line MDA-MB-468 (10, 11). Only in mouse keratinocytes were epithelin 1 and 2 shown to have distinct biological activities since epithelin 1 stimulated the growth of keratinocytes whereas epithelin 2 inhibited the action of epithelin 1 (10). cDNAs encoding epithelins and granulins were independently isolated showing that the cDNAs encoding the 6-kDa granulins and epithelins were identical. (7, 8). In both cases, the cDNA encoded a 63.5 kDa protein containing a 17-amino acid signal peptide which is absent from the amino acid sequence of secreted PCDGF (6) and seven and a half predicted internal sequence repeats of about 50 amino acids corresponding to the epithelins/granulins sequences. Presence of several glycosylation sites was also predicted from the deduced amino acid sequences (7, 8). It was proposed that epithelins and granulins were synthesized as an immature precursor that was processed into 6-kDa biologically active polypeptides (8). A 68-kDa protein called acrogranin similar to the precursor of epithelins/granulins had been isolated from the acrosomal compartment of the guinea pig (12). The function of this polypeptide remains to be elucidated. In addition, epithelial cell specific transforming growth factor (TGF-e), a 20-kDa polypeptide with a N-terminal sequence identical to human granulin A, was purified from bovine kidney (13). It is not clear at this time whether TGF-e is the partially processing product of epithelins/granulins precursor or is encoded by another gene. The study with PC cells provided the first evidence that the unprocessed precursor had biological activity and was acting as a growth modulator (6).

Growth factors regulate cell proliferation and differentiation by binding to specific cell surface receptors which, once activated, will mediate a cascade of second messengers pathways leading to biological cellular responses. Therefore, it is very important to characterize the interaction of newly characterized growth modulators to cell surface binding sites in cells where they exert biological response. Since PCDGF is a novel growth factor, experiments were carried out to investigate the properties of its binding to cells.

It has been shown that epithelin/granulin precursor mRNA is expressed by many tissues and cells in culture (7). Thus we first screened several cell lines to find one that did not express significant level of PCDGF so that binding studies would not be hampered by possible occupancy of cell surface binding sites by secreted PCDGF. We found that the mink lung epithelial cell line CCL64 expressed extremely low level of PCDGF mRNA and PCDGF inhibited the proliferation of CCL64 in a dose-dependent manner. Thus initial ^{125}I -PCDGF binding studies were performed using CCL64 cells. In this paper, we report results about the bio-

chemical analysis and identification of PCDGF cell surface binding sites for these cells and also for other cell lines.

MATERIALS AND METHODS

Expression of PCDGF in insect cells and purification of PCDGF. PCDGF cDNA was ligated into the EcoRI-SmaI sites of Baculovirus transfer vector pVL1392 (Invitrogen, San Diego, CA). Plasmid of pVL1392-PCDGF was used to cotransfect insect cells (SF9) with Baculovirus DNA (BaculoGold, PharMingen, San Diego, CA) and Baculovirus-PCDGF was selected according to the method of Summers (14). After infection of Baculovirus-PCDGF the insect cells (SF9 or High5) were cultured in Grace's medium for 48 hours at 27°C. The conditioned medium was collected and rPCDGF was purified by immunoaffinity chromatography on an anti-PCDGF antibody sepharose column. Details of the purification procedures are reported elsewhere.¹

Iodination of PCDGF. Affinity purified PCDGF was iodinated by the chloramine T method at 4°C. Briefly, 1 μg of PCDGF was incubated for 2 min with Na^{125}I (100 μCi) that had been preincubated for 90 seconds with 2 μg chloramine T (Sigma, St. Louis, MO). The reaction was quenched with the addition of 100 μl saturated tyrosine, 10 μl of a solution of 1% BSA and 2 μg sodium metabisulfite. After addition of 100 μl PBS, the iodinated protein was separated from free Na^{125}I by gel filtration on a Sephadex-G50 column (Pharmacia, Uppsala, Sweden) that had been preblocked with PBS containing 1% BSA then extensively washed with PBS. The labeled proteins were eluted with PBS and fractions monitored for radioactivity. Amount of incorporated radioactivity was estimated by TCA precipitation. Specific activity of ^{125}I -PCDGF was typically 30-50 $\mu\text{Ci}/\mu\text{g}$. Labeling conditions to reach higher specific activity always led to loss of biological activities for the radiolabeled protein.

^{125}I -PCDGF binding to CCL64 cells. The binding assays were performed using cells in suspension. Mink lung epithelial cell line, Mv1Lu (CCL64) (15) was obtained from the American Type Culture Collection (Rockville, MD). CCL64 cells were cultivated as monolayers in DME medium supplemented with 10% fetal bovine serum (FBS) until they reached confluence. At that time, cells were washed with PBS and detached by brief incubation with a solution of 0.25 mg/ml of trypsin and 1 mM EDTA. The cells were harvested by centrifugation, extensively washed with culture medium containing 1% BSA and proteinase inhibitors and counted with a hemocytometer. For binding assays, 10^6 cells were resuspended in 500 μl of binding buffer consisting of DME medium supplemented with 1% BSA in 1.5 ml Eppendorf tubes. Equilibrium binding was achieved by incubating CCL64 cells for 2 h at 22°C with 10⁵ cpm of ^{125}I -PCDGF and increasing concentrations of unlabeled PCDGF from 0 to 100 ng/ml. At the end of the incubation period, binding was stopped by centrifugation followed by 3 successive washings at 4°C with ice-cold binding buffer followed by centrifugation. Cell pellets were counted with a LKB gamma counter. Scatchard analysis of binding data was done by using the LIGAND computer program. Values described in the result section correspond to the average of three separate experiments each performed with duplicate determinations per experimental point.

Crosslinking studies of PCDGF to various cell lines. 3T3 fibroblasts were cultivated in DMEM-F12 medium supplemented with 10% CS and PC cells were cultivated in defined medium as described previously². PC cells were detached by brief incubation with PBS containing 1 mM EDTA. 3T3 fibroblasts were detached by brief exposure to trypsin solution. In both cases, the cells were washed extensively with DMEM-F12 medium supplemented with 1% BSA prior to doing the binding assay. For crosslinking studies, 5×10^5 cells were resuspended in 250 μl of binding buffer in Eppendorf tubes. ^{125}I -PCDGF was added in 50 μl of binding buffer with or without

¹X. Xia and G. Serrero, manuscript in preparation.

100-fold excess unlabeled competing ligand. Binding was performed as described in the previous paragraph. At the end of the incubation period, the cells were washed twice with 0.2% BSA-DME and once with PBS before crosslinking was carried out. The cells were resuspended in 200 μ l PBS containing 1 mM disuccinimidyl suberate (DSS, Pierce, Rockville, IL) which had been prepared at a stock concentration of 100 mM in DMSO just prior to being used. Incubation was carried out at room temperature for 20 min. After crosslinking, 10 μ l of 1 M Tris-HCl buffer (pH 7.4) was added to quench the reaction. The cells were centrifuged, washed and extracted with 25 μ l extraction buffer (PBS containing 1% Triton X-100, 0.1% SDS, 0.5 mM phenylmethylsulfonyl fluoride (PMSF)) at 4°C for 1 h. Samples were centrifuged for 5 min at 13,000 $\times g$ and 25 μ l of supernatant from each sample was mixed with 4 μ l of 20% SDS and 15 μ l 3 \times Laemmli's sample buffer (16) containing β -mercaptoethanol and boiled for 5 min. Electrophoresis of samples was carried out on 7% SDS polyacrylamide slab gel according to Laemmli (16) using a mini-gel apparatus (Bio-Rad, Richmond, CA). The dried gels were exposed to X-ray films for autoradiography at -70°C.

RESULTS

Binding and Scatchard Analysis of 125 I-PCDGF to CCL64 Cells

Binding of 125 I-PCDGF to CCL64 cells was examined. PCDGF was iodinated with chloramine T as described in the method section. Preliminary experiments established that 125 I-PCDGF bound to CCL64 cells in a time-dependent manner, with maximal specific binding obtained at 22°C for 2 h (data not shown). To measure 125 I-PCDGF binding, CCL64 cells were incubated with labeled tracer in the presence of increasing amounts of unlabeled PCDGF. 2.8% of added 125 I-PCDGF bound to the cells in the absence of unlabeled

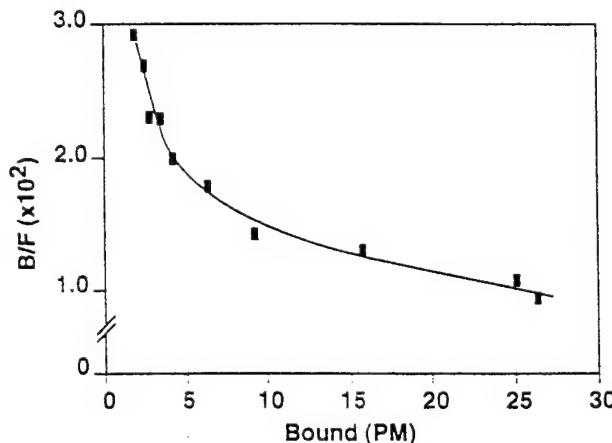


FIG. 1. Scatchard analysis of 125 I-PCDGF binding to CCL64 cells. CCL64 cells were incubated for 2 h at 22°C with 125 I-PCDGF (10^5 cpm/tube) in the presence of increasing concentrations of unlabeled PCDGF (0-100 ng). The cells were washed three times and cell-associated radioactivity was measured in a gamma counter. Nonspecific binding was determined in the presence of a 200-fold excess of unlabeled PCDGF. Scatchard analysis of PCDGF binding data was done using LIGAND computer program. Scatchard representation of one of three experiments is shown here.

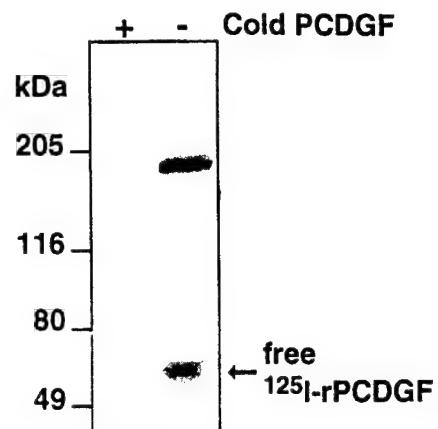


FIG. 2. Affinity labeling of 125 I-PCDGF binding sites on CCL64 cells. 125 I-PCDGF was incubated with 5×10^5 CCL64 cells in the presence or absence of 100-fold excess of unlabeled PCDGF for 2 h at 22°C and was crosslinked to its receptor with 1 mM DSS (final concentration) for 20 min at 4°C. The crosslinking reaction was quenched by the addition of Tris-HCl buffer (pH 7.4), and the cells were extracted by 1% Triton X-100. The supernatant was mixed with 3 \times SDS-PAGE sample buffer containing β -mercaptoethanol and electrophoresed on a 7% polyacrylamide gel. After electrophoresis, the gel was dried and exposed to the X-ray film.

competitor. A concentration-dependent inhibition of iodinated PCDGF binding was observed in the presence of unlabeled PCDGF. A 90% inhibition of total binding was observed at the highest concentration of unlabeled PCDGF tested (300 ng/ml).

Equilibrium binding data were analyzed by the LIGAND computer program (17). Scatchard representation from one typical experiment is shown in Fig. 1. The data indicated that binding of 125 I-PCDGF to CCL64 cells was curvilinear corresponding to the presence of two classes of cell surface receptors for PCDGF. Based on three independent experiments, binding parameters calculated were $K_{d1} = 43 \pm 15$ pM with 560 ± 170 sites/cell for the high affinity sites, and $K_{d2} = 3.9 \pm 1.9$ nM with 16350 ± 5900 sites/cell for the low affinity sites.

Crosslinking of 125 I-PCDGF to CCL64 Cells

125 I-PCDGF bound to CCL64 cells was crosslinked by the addition of the chemical crosslinker DSS. Crosslinked material was separated by electrophoresis on 7% polyacrylamide gels followed by analysis by autoradiography. As shown in Fig. 2, autoradiographic analysis revealed the presence of one major crosslinked band with an apparent molecular weight of 190 kDa. The intensity of the radiolabeled crosslinked band was significantly decreased in the lanes where binding was carried out in the presence of excess cold PCDGF, showing that unlabeled PCDGF competed with 125 I-PCDGF. Crosslinked band could not be detected if experiment was performed in the absence of crosslinker DSS and

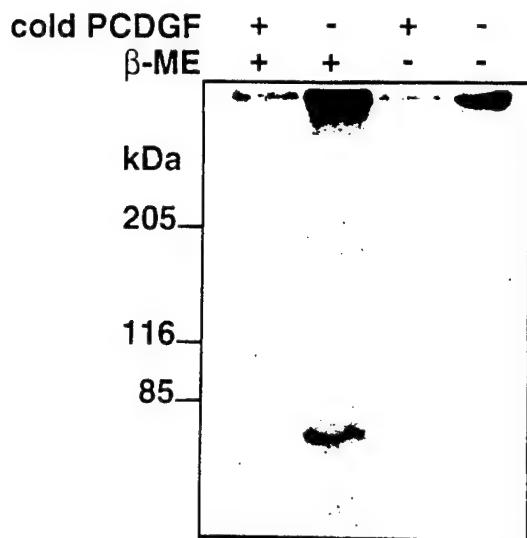


FIG. 3. Comparison of affinity labeling of ^{125}I -PCDGF to CCL64 cells in reducing and non-reducing conditions. Binding and crosslinking of ^{125}I -PCDGF to CCL64 cells were done as described previously. The extracts were mixed with SDS-PAGE sample buffer in the presence or absence of 10 mM β -mercaptoethanol (β -ME) and separated on a 7% polyacrylamide gel. After electrophoresis, the gel was dried and exposed to the X-ray film. In non-reducing condition the migration of PCDGF in SDS-PAGE was changed due to the high cysteine content of the protein.

when using cells which were not responsive to PCDGF (data not shown). As shown in Fig. 3, the molecular weight of the crosslinked band was not significantly changed whether samples were treated or not with β -mercaptoethanol prior to performing the electrophoresis. These data would suggest that if the receptors are multimeric, they are not linked by disulphide bridges.

PCDGF Receptors Are Ubiquitously Expressed in Cells of Different Embryonic Origin

We have shown previously that PCDGF is a growth stimulator for mouse 3T3 fibroblasts and for the highly tumorigenic teratoma-derived PC cell line from which PCDGF was originally purified (6). Binding and crosslinking experiments of ^{125}I -PCDGF to both cell types were then performed to determine the size of crosslinked PCDGF receptors on both types of cells. SDS-PAGE of the crosslinked products indicated the presence of a major wide labeled band which migrated with an apparent molecular weight similar to the ones in CCL64 cells. The formation of crosslinked bands was prevented by addition of unlabeled PCDGF, showing the binding of ^{125}I -PCDGF to these cells was specific (Fig. 4). Because PC cells were cultivated in defined medium in the absence of serum, these results indicate that the crosslinked band does not correspond to a possible interaction between PCDGF and a binding protein present in serum. These data suggest that PCDGF cell surface receptors are expressed in many

different types of cells responsive to PCDGF and the molecular weight of these receptors on these different cells is similar.

DISCUSSION

As a member of a new growth factor family, PCDGF may play an important role in cellular proliferation and differentiation. The characterization of receptors which mediate the effects of growth factors such as PCDGF is of importance due to the possible involvement of these proteins in physiological and/or pathological processes. The data presented here investigate for the first time the binding of PCDGF (epithelin/granulin precursor) to cell surface receptors. Our experiments show that PCDGF can be iodinated and that ^{125}I -PCDGF specifically binds to several cell types. Scatchard analysis of the equilibrium binding data with the epithelial cell line CCL64 indicates the existence of two classes of high and low affinity PCDGF receptors. Crosslinking experiments reveals the presence of one major class of binding sites for PCDGF on CCL64 with the molecular weights of about 120 kDa. Furthermore, it is shown that binding sites for PCDGF are present on several PCDGF-responsive cell lines in addition to CCL64 cells.

The existence of two classes of high and low affinity receptors for PCDGF by Scatchard analysis suggests either possible interactions between identical binding sites, or multiple independent sites. However, it is now

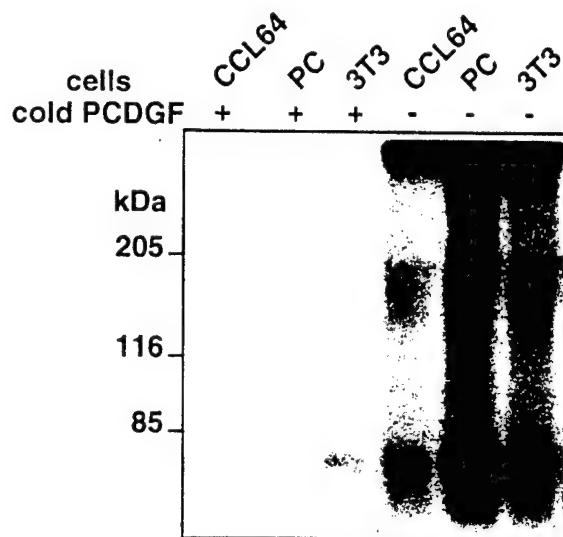


FIG. 4. Presence of PCDGF binding sites on the cell surface of several cell lines. 3T3 fibroblasts, PC cells and CCL64 cells were prepared as described under Materials and Methods. Cell suspensions were incubated for 2 h at 22°C with ^{125}I -PCDGF in the presence or absence of unlabeled PCDGF. After incubation, the cells were treated with DSS for 20 min at 4°C and extracted in 1% Triton X-100. The extracts were analyzed by SDS-PAGE and autoradiography performed as mentioned before.

well documented for other growth factors that the formation of high affinity sites may be the result of receptor subunit interaction, such as homodimerization of low affinity receptors as it has been shown with EGF receptor (18), heterodimerization of α and β chains as shown for interleukin-2 receptor (19), or heterodimerization of a low affinity receptor with an affinity converter subunit as recently reported between the receptors for interleukin-6, leukemia inhibitory factor, oncostatin M, ciliary neurotrophic factor and the gp130 protein (20, 21). Crosslinking experiments of ^{125}I -PCDGF binding to cell surface binding sites revealed the presence of a single band of 190-kDa after SDS-PAGE and autoradiography. This does not necessarily imply that only a single binding species is present.

Like many other cytokines and growth factors (20, 22, 23), PCDGF may be multifunctional and exhibit pleiotropic biological effects on cells. To date, the only physiological role known to PCDGF or epithelins is related to cell proliferation. Unlike epithelins, PCDGF affects proliferation both on epithelial and non-epithelial cells (6). Dependent on the cell types, PCDGF either stimulates or inhibits the cellular proliferation. The effective concentration of PCDGF for inhibition or stimulation on the cellular proliferation was similar.⁵ An interesting question remained to be answered is whether the different biological effects of PCDGF are mediated by the different classes of binding sites independently or by the diversified downstream signal pathways in cells.

PCDGF is the precursor of epithelins/granulins. Up to now there is only one report about the characterization of receptor for epithelins (11). In addition, the binding sites on epithelial cells for TGF- β , a protein with the N-terminal sequence identical to the human granulin A, is reported (24). Scatchard analysis revealed there were two different binding sites with different affinity for epithelins and results from crosslinking showed there was a single band of ligand-receptor complex with about 145 kDa on the surface of MDA-MB-468. Furthermore, it is observed that epithelin 1, 2 and 3 seemed to share same receptor because they all specifically competed with iodinated epithelin 1 for the binding to the membrane. Despite the evidence that showed that PCDGF receptor is different from epithelins receptors, we cannot totally exclude the possibility that PCDGF and epithelins share same receptors or some classes of the receptors. Molecular cloning and expression of PCDGF receptors should enable us to study the exact nature of the interactions between PCDGF and epithelins/granulins receptors. Even if PCDGF receptors are different from epithelins receptors,

cross-talk and transmodulation between these receptors remain potentially important regulatory events to consider, as has been shown to occur between EGF receptor (25) and platelet-derived growth factor receptors (26).

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Stimulation of PC Cell-Derived Growth Factor (Epithelin/Granulin Precursor) Expression by Estradiol in Human Breast Cancer Cells

Runqing Lu* and Ginette Serrero*†¹

*Department of Pharmaceutical Sciences, University of Maryland School of Pharmacy, 20 North Pine Street, Baltimore, Maryland 21201; and [†]Program of Oncology, Marlene and Stewart Greenebaum Cancer Center, University of Maryland, Baltimore, Maryland 21201

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PC cell-derived growth factor (PCDGF) is an 88 kDa glycosylated protein isolated from a highly tumorigenic mouse teratoma derived cell line which is similar to the epithelin/granulin precursor. Using Northern blot and western blot analyses, we detect the expression of PCDGF mRNA and protein in MCF-7 human breast cancer cells. We show that 17- β -estradiol stimulates PCDGF mRNA and protein expression in a time and dose-dependent manner. The stimulation of PCDGF expression by 17- β -estradiol was observed as early as 4 hours and reached a maximum at 12 hours. Maximal stimulation of PCDGF mRNA and protein expression by 17- β -estradiol was observed at a concentration of 10⁻⁸ M. The stimulation of PCDGF expression by 17- β -estradiol was completely inhibited by treatment with actinomycin D and with the antiestrogen 4-hydroxytamoxifen. The stimulation of PCDGF expression was also demonstrated in another human estrogen-responsive cell line T47D. The results presented here provide evidence of a novel estradiol responsive gene product in human breast cancer cell lines and give information about the hormonal control of epithelin/granulin (PCDGF) expression in these cells. © 1999 Academic Press

PC cell derived growth factor is an 88 kDa glycosylated protein purified from the highly tumorigenic teratoma-derived cell line PC 1. Structural analysis of PCDGF indicated that it consisted of a 68 kDa core

¹ To whom correspondence should be addressed. Fax: 410 706-0346. E-mail: gserre@pharmacy.umd.edu

Abbreviations used: E: 17- β -estradiol; ER: estrogen receptor; ERE: estrogen response element; FBS: fetal bovine serum; α -MEM: phenol-red free α -modified Eagle's medium; PCDGF: PC cell-derived growth factor; PFMEM: α -MEM supplemented with 5% charcoal-stripped FBS.

protein and a 20 kDa carbohydrate moiety (1). Amino-acid and cDNA sequencing indicated that PCDGF cDNA was identical to the epithelin/granulin precursor (2-3). Epithelin and granulins are 6 kDa double cysteine-rich polypeptides originally purified from rat kidney (4) and from human granulocyte extracts (5) characterized by a unique highly conserved motif (6). These polypeptides have subsequently been identified throughout the entire vertebrate kingdom (6). Epithelins have been shown to act as dual growth effectors that promote or inhibit the growth of various mammalian cells in culture including keratinocytes and human epidermoid carcinoma cell line A431 (4). Interestingly, epithelin 2 can antagonize the mitogenic action of epithelin 1 (4) on keratinocyte whereas they both inhibit proliferation of human breast cancer cells (7). Cloning of cDNA for epithelins and granulins has shown that they are encoded by a 593 amino-acids common precursor called epithelin/granulin precursor containing a secretory signal peptide and seven and a half repeats of the cysteine rich 6 kDa motifs (4-5). Although it was postulated that epithelin/granulin precursor needed to be processed into biologically active 6 kDa polypeptides (4), several examples have been reported of the biological importance of the precursor. Acrogranin, a 67 kDa glycoprotein found in the acrosomal compartment of the sperm head has an amino-terminal sequence identical to the precursor (8). The 88 kDa glycoprotein PC-cell derived growth factor was shown to stimulate the proliferation of fibroblasts cells (1). Moreover, the granulin/epithelin precursor also stimulated the growth of mouse embryo fibroblasts null for the type 1 insulin-like growth factor receptor (9). In this paper, we investigated the expression of PCDGF in human breast cancer cell lines and showed that its expression is stimulated by estradiol.

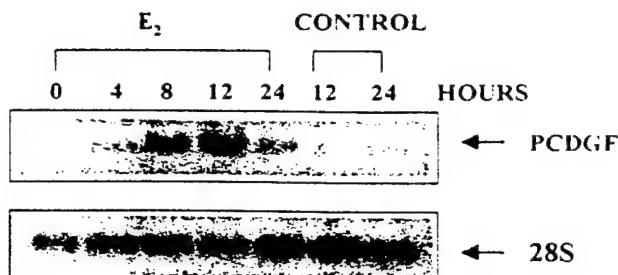


FIG. 1. Time-course of E_2 effect on PCDGF mRNA expression. MCF-7 cells were maintained in steroid-free conditions by being cultured in PFMEM medium for 24h as described in the method section. Cells were then treated with 10^{-9} M E_2 . Control cells were treated with the same volume of ethanol only (0.1%). Total RNA was extracted at the indicated times. PCDGF expression was measured by Northern blot analysis. 28S ribosomal RNA expression was measured as internal control for equal RNA loading.

MATERIALS AND METHODS

Materials. 17 β -estradiol (E_2), 4-OH-tamoxifen, cycloheximide, actinomycin D were obtained from Sigma. Protein A-Sepharose was from Pharmacia. Culture media and fetal bovine serum (FBS) and Trizol reagent were purchased from Life Technology. Tissue culture plasticware was supplied by Corning Incorporated.

Cell culture. Human breast cancer MCF-7 cells were obtained from the American Type Culture Collection (ATCC) and T47D cells (ATCC) were kindly provided by Dr. Angela Brodie (University of Maryland). Both cell lines were cultivated in Dulbecco's modified Eagle's medium-Ham's F-12 medium (1:1 mixture) supplemented with 5% FBS. For all experiments, cells were cultivated as described before in 60-mm dishes. After reaching 70% confluence, cell monolayers were washed twice with phenol-red free α -modified Eagle's medium (α -MEM) and incubated for 24 hours in α -MEM supplemented with 5% charcoal-stripped FBS (PFMEM). Cells were then washed twice again with α -MEM and then incubated in PFMEM for different times in the presence and absence of various concentrations of agents to be assayed, as indicated in the figure legends.

RNA isolation and Northern blot analysis. Total RNA was extracted using Trizol reagent from duplicate 60-mm dishes. 20 μ g total RNA were used to study PCDGF mRNA expression by Northern blot analysis using a human PCDGF cDNA probe carried out as described previously (10). The signals obtained by autoradiography were quantified by densitometric analysis and normalized to the level of 28S ribosomal RNA internal control.

Western blotting. MCF-7 cells were lysed in 1 ml PBS buffer containing 1% Triton X-100 with a protease inhibitor cocktail consisting of 200 μ M PMSF, 1 μ M leupeptin, 0.5 μ M aprotinin and 1 mM EDTA from duplicate 60 mm dishes. Cells lysates and conditioned media from 4×10^6 cells were incubated overnight at 4°C with 5 μ l of rabbit anti-human PCDGF polyclonal antibody. The immunocomplexes were then collected by incubation with 50 μ l of protein A-Sepharose slurry for 4 hours. The Sepharose beads were washed three times with cold PBS and then boiled in 2 x reducing SDS sample buffer (2% SDS, 10% glycerol, 62 mM Tris-HCl pH 6.8, 1% β -mercaptoethanol) and loaded on a 10% SDS-polyacrylamide gel. Proteins were then electrophoretically transferred to a 0.2 μ m PVDF membrane (Millipore) at 100 V for 1 hour. The blot was blocked overnight at 4°C in 5% skim milk and then probed with the anti-PCDGF polyclonal antibody for 1 hour at room temperature in PBST buffer (PBS buffer containing 0.05% Tween 20 plus 1% skim milk). After washing three times, 5 min each in PBST, the blot was incubated at room temperature with goat anti-rabbit IgG conjugated to

horseradish-peroxidase (KPL) for 1h in PBST containing 1% skim milk. The washing step was repeated twice. Finally, immunoreactivity on the blot was visualized by the enhanced chemiluminescence detection system (Amersham). All experiments were repeated at least three times.

RESULTS

Time-dependent stimulation of PCDGF mRNA expression by E_2 . MCF-7 cells were cultivated in phenol-red free α -MEM medium supplemented with 5% charcoal-stripped FBS in the presence or absence of E_2 (10^{-9} M). Total RNA was extracted at indicated times to measure PCDGF mRNA expression by Northern blot analysis (Fig. 1). Results showed that MCF-7 cells expressed PCDGF mRNA and that PCDGF mRNA expression increased in a time-dependent fashion upon treatment with E_2 . Densitometric analysis of the data indicated that the increase in PCDGF mRNA expression was 1.5-fold after 4 hours of exposure to E_2 , reached a 5-fold maximum induction \pm 12 hours and decreased to 2-fold above basal level \pm 24 hours. PCDGF mRNA expression in control untreated cells remained at a low level throughout the same period.

Dose-dependent stimulation of PCDGF mRNA expression by E_2 . MCF-7 cells were treated with different concentrations of E_2 (10^{-14} to 10^{-8} M) for 12h followed by RNA extraction and Northern blot analysis. As shown in Fig. 2A, treatment of MCF-7 cells with increasing concentrations of E_2 resulted in a dose-dependent increase in the level of PCDGF mRNA expression. The stimulation of PCDGF mRNA expression by E_2 was observed at concentrations as low as 10^{-12} M and was maximal with a 5-fold stimulator of PCDGF

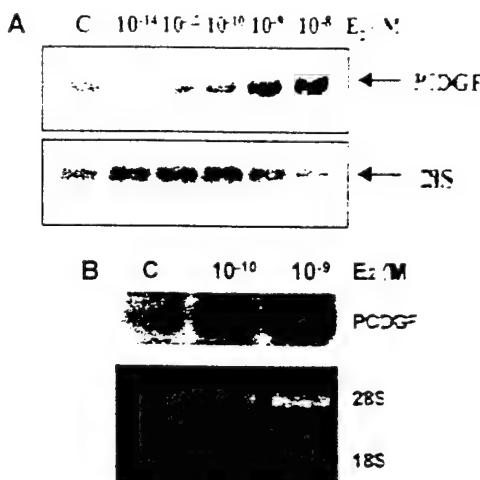


FIG. 2. Effect of increasing concentrations of E_2 on PCDGF mRNA expression. MCF-7 and T47D cells at 75% confluence were cultivated in PFMEM medium for 24h as described above. Cells were then treated with the indicated concentrations of E_2 . Control cells were treated with the same volume of ethanol (0.1%). Total RNA was extracted 12h later. PCDGF mRNA expression was measured by Northern blot analysis. **A** MCF-7 cells. **B** T47D cells.

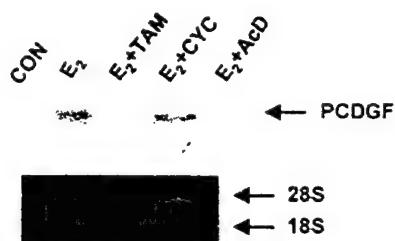


FIG. 3. Effect of 4-OH-tamoxifen and actinomycin D on the stimulation of PCDGF mRNA expression by E_2 . MCF-7 cells cultivated in estrogen-depleted medium for 24h were treated for 6h with 10^{-9} M E_2 alone or in the presence of one of the following three compounds: 4-OH-tamoxifen (TAM, 1 μ M), Actinomycin D (AcD, 5 μ g/ml) and Cycloheximide (CYC, 10 μ g/ml). Control cells (CON) not treated with E_2 but with the same volume of ethanol (0.1%) were used as negative control. Total RNA was collected to examine the level of expression of PCDGF mRNA by Northern blot analysis.

expression in cells treated at 10^{-8} M E_2 , a concentration known to maximally stimulate expression of estrogen-responsive genes such as progesterone receptor in MCF-7 cells (11).

The induction of PCDGF mRNA expression was also observed in another ER-positive cell line T47D with a four-fold stimulation of PCDGF mRNA expression at 10^{-9} M E_2 (Fig. 2B).

Effect of Tamoxifen on PCDGF mRNA stimulation by E_2 . Experiments were carried out to examine whether the stimulatory effect of PCDGF expression was inhibited by treatment with the antiestrogen 4-OH-Tamoxifen. We used a concentration of 1 μ M of 4-OH-tamoxifen which has strong antagonist activity with no partial agonist activity (12). As shown in Fig. 3, treatment of MCF-7 cells with 4-OH-tamoxifen blocked the increase of PCDGF expression by E_2 (10^{-9} M). These data suggest that the effect of E_2 on PCDGF expression is mediated by estrogen receptor.

We then examined the effect of protein synthesis inhibitor cycloheximide (10 μ g/ml) and RNA synthesis inhibitor actinomycin D (5 μ g/ml) on the stimulation of PCDGF mRNA expression by short-term treatment with E_2 (10^{-9} M). As shown in Fig. 3, the stimulatory effect of E_2 on PCDGF mRNA expression was completely abolished by treatment with actinomycin D but not with cycloheximide. These data suggest that the stimulation of PCDGF expression by E_2 requires *de novo* mRNA synthesis.

PCDGF protein expression is stimulated by E_2 . Cell lysates and conditioned media of MCF-7 cells were collected at various times after treatment with E_2 (10^{-9} M) to examine PCDGF protein expression using immunoprecipitation and western blot analysis with anti-human PCDGF antibody as described in the materials and methods section. As shown in Fig. 4, treatment of MCF-7 cells with E_2 resulted in a time-dependent stimulation of PCDGF expression in cell lysates, reaching a

maximum at 12h. E_2 treatment also caused the accumulation of PCDGF protein in conditioned medium. The cell lysates and conditioned media of control cells (0.1% ethanol only) showed a low, stable level of PCDGF protein expression throughout the same period (data not shown).

DISCUSSION

PC cell derived growth factor (PCDGF) is an 88 kDa glycoprotein corresponding to the precursor of the 6 kDa double-cysteine rich polypeptides called epithelins or granulins (1-4). We show here that human mammary carcinoma cells MCF-7 and T47 D express PCDGF mRNA and protein and that the 88 kDa precursor protein is secreted in their culture medium. Data presented here demonstrate that estrogen stimulates PCDGF expression in MCF-7 cells in a time and dose-dependent fashion. E_2 also stimulates the time-dependent production of PCDGF protein in a pattern similar to the stimulation of PCDGF mRNA expression. The stimulation of PCDGF expression by E_2 is not restricted to MCF-7 cells since it was also demonstrated in another estrogen responsive cell line T47D. PCDGF mRNA and protein were also detected in the ER-negative human breast cancer cells MDA-MB-468 and MDA-MB-453 but the level of PCDGF expression was not stimulated by estradiol treatment (Lu and Serrero, unpublished result).

The fact that 4-hydroxytamoxifen abolishes the stimulatory effect of E_2 suggests that the induction of PCDGF expression is mediated via an estrogen receptor (ER)-related pathway.

The promoter regions of mouse and human epithelin/granulin precursor have been characterized (13-14). Analysis of the sequence failed to indicate the presence of a classical estrogen responsive element (ERE) in the proximal region of the precursor promoter (6), suggesting that any steroid effect may be indirect. However, very few of the human mRNAs known to be directly regulated by estrogen in mammary epithelial cells have been shown to be induced from a canonical ERE

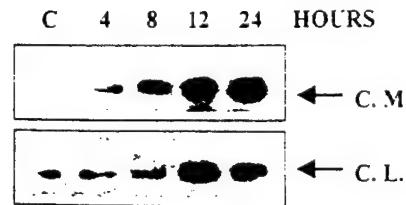


FIG. 4. PCDGF protein expression in MCF-7 cell lysates and conditioned media during incubation with E_2 . MCF-7 cells were cultivated in PFMEM medium for 24h. The medium was removed and replaced with fresh PFMEM medium. Cell lysates (C.L.) and conditioned media (C.M.) were collected at indicated times after incubation with 10^{-9} M E_2 , and Western blot analysis of PCDGF expression was performed as described in the materials and methods section.

(15). Most estrogen-responsive genes identified to date contain one or more imperfect EREs or multiple copies of an ERE half-site rather than a classical ERE (16–17). Such ERE half sites have been found in PCDGF promoter region (13–14). Detailed promoter studies should allow to explore this question.

The intact 88 kDa epithelin/granulin precursor, also called PCDGF, was originally purified from the conditioned medium of a teratoma derived cell line of mesenchymal origin (1). The data presented here show that cells of epithelial origin can also secrete the intact precursor. The expression of the precursor in epithelial cells as well as in fibroblasts would suggest a widespread role for PCDGF in contrast to some other growth factors that have a more restricted distribution. Receptors for the 6 kDa epithelin, the processed form of PCDGF have been characterized on human mammary epithelial carcinoma cells MDA-MB-468 by Scatchard analysis of binding studies and chemical cross-linking of ¹²⁵I-labeled epithelin (7). Two classes of binding sites with an apparent molecular weight of 145 kDa were reported on these cells. Cell surface binding sites for ¹²⁵I-PCDGF with an apparent molecular weight of 120 kDa have also been characterized by Scatchard analysis and by affinity labeling in several cell lines of mesenchymal and epithelial origins including mouse mammary epithelial cells (18). The fact that mammary epithelial cells secrete the epithelin/granulin precursor is interesting as it raises questions about the possible role of various members of the same family of polypeptide growth factors in these cells. Our preliminary results indicate that PCDGF is a mitogen for mouse and human mammary epithelial cells (Xia, Lu and Serrero, unpublished result). Experiments are performed to examine in detail the role of PCDGF in the growth of human mammary epithelial cells.

In summary, the results presented here provide novel information about the hormonal control of epithelin/granulin expression and identify a novel gene regulated by estradiol in mammary epithelial cells.

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Resveratrol, a Natural Product Derived From Grape, Exhibits Antiestrogenic Activity and Inhibits the Growth of Human Breast Cancer Cells

RUNQING LU¹ AND GINETTE SERRERO^{1,2*}

¹Department of Pharmaceutical Sciences, University of Maryland School of Pharmacy, Baltimore, Maryland

²Program of Oncology, University of Maryland Marlene and Stewart Greenebaum Cancer Center, Baltimore, Maryland

Resveratrol is a natural phytoalexin compound found in grapes and other food products. In this study, the effect of resveratrol on the growth of human breast cancer cells was examined. Results show that resveratrol inhibits the growth of estrogen receptor(ER)-positive MCF-7 cells in a dose-dependent fashion. Detailed studies with MCF-7 cells demonstrate that resveratrol antagonized the growth-promoting effect of 17-β-estradiol (E₂) in a dose-dependent fashion at both the cellular (cell growth) and the molecular (gene activation) levels. At 5×10^{-6} M, resveratrol abolished the growth-stimulatory effect mediated by concentrations of E₂ up to 10^{-4} M. The antiestrogenic effect of resveratrol could be observed at a concentration of 10^{-6} M and above. The antiestrogenic effect of resveratrol was also demonstrated at the molecular level. Resveratrol in a dose-dependent fashion antagonized the stimulation by E₂ of progesterone receptor gene expression in MCF-7 cells. Moreover, expression of transforming growth factor-α and insulin-like growth factor I receptor mRNA was inhibited while the expression of transforming growth factor β2 mRNA was significantly elevated in MCF-7 cells cultivated in the presence of resveratrol (10^{-5} M). In summary, our results show that resveratrol, a partial ER agonist itself, acts as an ER antagonist in the presence of estrogen leading to inhibition of human breast cancer cells. *J. Cell. Physiol.* 179: 297-304, 1999. © 1999 Wiley-Liss, Inc.

Resveratrol (3,5,4'-trihydroxystilbene) is a bioflavonoid found in many plants, including grapes and mulberries. In the plant world, resveratrol, regarded as an antibiotic, is thought to play an important role in the host defense mechanism against infection and injury (Derckx and Creasy, 1989). Red wine is believed to be the main source of resveratrol in the human diet. Recent studies have associated resveratrol with the cardioprotective effect observed among people with moderate wine consumption. For example, it has been reported that resveratrol had protective effects against oxidation of lipoproteins (Frankel et al., 1993), an important step in atherogenesis. It also inhibited platelet aggregation and altered eicosanoid synthesis (Soleas et al., 1997). Moreover, resveratrol has been found to possess chemopreventive activity by inhibiting ribonucleotide reductase (Fontecave et al., 1998) and cellular events associated with cell proliferation, tumor initiation, promotion, and progression (Jang et al., 1997; Mgbonyebe et al., 1998).

Bioflavonoids are major constituents in plants and vegetables (Harborne, 1994). Some of them have been categorized as phytoestrogens because these environmentally derived compounds bind and activate the es-

trogen receptor (ER) although they are less active than endogenous estrogens (McLachlan, 1995). Recent studies have also suggested that some of the flavonoids have an antiestrogenic effect by preventing more potent endogenous estrogen from binding to the ER, similarly to tamoxifen (Makela et al., 1995a,b; Markaverich et al., 1995; Collins et al., 1997). For example, phytochemicals such as enterolactone, narigenin, and phloretin have all been shown to possess mixed agonist/antagonist activity of the ER (Mousavi and Adlercreutz, 1992; Miksicek, 1993; Ruh et al., 1995). Indeed, the chemopreventive effect of bioflavonoids has been used to explain the low incidence of breast cancer and prostate cancer among vegetarians and Orientals who

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*Correspondence to: Ginette Serrero, Department of Pharmaceutical Sciences, University of Maryland School of Pharmacy, 20 N. Pine street, Baltimore, MD 21201. E-mail: gserrero@pharmacy.ab.umd.edu

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normally have higher blood levels of phytoestrogens (Setchell and Adlercreutz, 1988; Adlercreutz et al., 1992; Makela et al., 1995). Recently, Gehm et al. (1997) have reported that resveratrol can bind and activate ER and stimulate the growth of ER-positive breast cancer cells. However, the effect of resveratrol on the growth of ER-positive breast cancer cells in the presence of E_2 had not been thoroughly studied. In the present study, we examined the effect of resveratrol either alone or in combination with estrogen on the growth of MCF-7 breast cancer cells at both cellular and molecular levels.

MATERIALS AND METHODS

Cell culture

MCF-7 cells obtained from the American Type Culture Collection (ATCC, Manhassas, VA) were maintained in DME-F12 medium (1:1 mixture of Dulbecco's modified Eagle's medium [DMEM] and Ham's F12 medium) supplemented with 5% fetal bovine serum (FBS; Life Technologies, Gaithersburg, MD).

Growth of MCF-7 cells cultivated in DME-F12 medium supplemented with 5% FBS

To determine the effect of resveratrol on cell growth, MCF-7 cells were plated in 6-well plates at 10^5 cells per well in 2 ml of DME-F12 medium supplemented with 5% FBS in the presence or absence of increasing concentrations of resveratrol (Sigma, St. Louis, MO). The cell number was measured every 2 days till day 6 with a hemocytometer after detaching the cells with trypsin-EDTA (Life Technologies).

Growth of MCF-7 cells in estrogen-depleted medium

To examine the effect of resveratrol and 17β -estradiol (E_2) (Sigma) on cell growth, MCF-7 cells were plated in 24-well plates at 5×10^4 cells per well in 1 ml of phenol-red free α -modified Eagle's medium (α -MEM; Life Technologies) supplemented with 5% charcoal-stripped FBS (PFMEM) for 24 hr. The charcoal-stripped FBS was prepared by double-stripping FBS with 1% charcoal (Sigma) and 0.5% Dextran T40 (Pharmacia, Piscataway, NJ) according to Katzenellenbogen et al. (1987). The medium was then removed and replaced with fresh PFMEM medium in the presence or absence of various concentrations of resveratrol and E_2 as indicated in the figures. Cells were counted on day 6 with a hemocytometer as described above.

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

mRNA expression for progesterone receptor (PR) and for several autocrine growth factors and their receptors was measured by RT-PCR of RNA prepared from MCF-7 cells treated with or without estradiol and resveratrol. For the study of PR mRNA expression, MCF-7 human breast cancer cells (3×10^5 cells per well) were plated in six-well plates in 2 ml of DME-F12 medium supplemented with 5% FBS. Two days later, the cells were washed and cultivated in estrogen-free PFMEM medium for 24 hr. MCF-7 cells were incubated with different concentrations of E_2 , resveratrol, and 4-OH-tamoxifen (Sigma) for 24 hr since the stimulation of PR

mRNA expression by E_2 reaches a maximum after 24 hr (May et al., 1989).

For the study of the expression of autocrine growth regulators such as transforming growth factor- α (TGF- α), transforming growth factor- β 1, - β 2, - β 3 (TGF- β 1, β 2, β 3), insulin-like growth factor I (IGF-I), insulin-like growth factor II (IGF-II), or growth factor receptors such as IGF-I receptor (IGF-IR), TGF- β receptors, and epidermal growth factor receptor (EGFR), MCF-7 cells were seeded at 10^5 cells per well in six-well plate in DME-F12 medium plus 5% FBS in the presence or absence of different concentrations of resveratrol for 6 days. Total RNA was isolated using TRIZOL reagent (Gibco, Life Technologies) according to the manufacturer's protocol. Specific mRNA expression was examined by RT-PCR using human β -actin mRNA expression as an internal control for RNA equal loading. The RT-PCR reaction in the exponentially amplifying cycle allows semiquantitative comparison of the mRNA expression (Ide et al., 1997). Briefly, 5 μ g each of total RNA from resveratrol-treated MCF-7 cells was reverse transcribed by random primer and Super-script II reverse transcriptase (Life Technologies). The resulting cDNA was subjected to PCR with the primers described below. The amplification reactions were performed with an initial incubation step at 94°C for 3 min followed by 25 cycles each (30 cycles for TGF- β 2, TGF- β) at 94°C for 1 min, 60°C for 45 sec, 72°C for 2 min. These cycles were followed by a final incubation step at 72°C for 7 min. The samples were subjected to electrophoresis in 1.2% agarose gel and stained with ethidium bromide. The identities of PCR products were confirmed by restriction enzyme digestion. The gels were blotted onto nylon membrane (BIO-RAD, Hercules, CA) in 10× SSC. Southern blot was conducted as described before (Eisinger and Serrero, 1993; Quinn et al., 1996) using corresponding sense primer for each PCR product as a probe. The probes were end labeled with [γ - 32 P] ATP using T4 polynucleotide kinase (Life Technologies). The RT-PCR analysis was repeated at least three times for each of the two independent experiments. The primers used for amplification of PR and IGF-IR were synthesized according to published sequences (Quinn et al., 1996; Hobisch et al., 1997). The primers for TGF- α , TGF- β 2, and β -actin were obtained from Clontech (Palo Alto, CA). The sizes of amplified PCR fragments were 297 bp for TGF- α , 755 bp for IGF-IR, 838 bp for β -actin, 415 bp for TGF- β 2, and 742 bp for PR.

Statistics

Experiments were carried out in triplicate and data were expressed as mean \pm SD. Two-tailed Student's *t*-test was used for statistical analysis of the data. $P < 0.05$ was taken as the level of significance.

RESULTS

Effect of resveratrol on the growth of MCF-7 cells cultivated in DME-F12 supplemented with 5% FBS

MCF-7 cells were plated in DME-F12 medium supplemented with 5% FBS in the presence of increasing concentrations of resveratrol. Control cells were treated with the same volume of vehicle only (0.1% ethanol). As shown in Figure 1, resveratrol inhibited the growth of MCF-7 cells in a dose-dependent fashion.

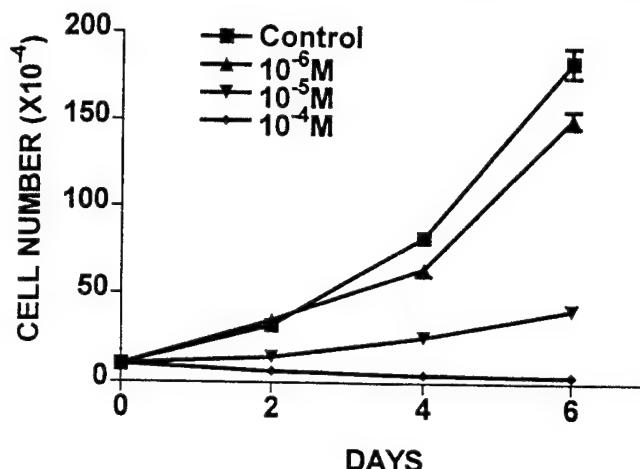


Fig. 1. Effect of resveratrol on the growth of MCF-7 cells cultivated in regular medium. MCF-7 cells were plated at 10^5 cells per well in 24-well plates in DME-F12 medium plus 5% FBS either in the absence (0.1% ethanol only) or in the presence of indicated concentrations of resveratrol. Cell number per well was determined every other day until day 6. Values are expressed as mean \pm SD of triplicate determinations.

Addition of 10^{-5} M resveratrol resulted in an 82% inhibition of MCF-7 cell growth after 6 days while at 10^{-6} M, only a 10% inhibition was observed. The cells treated with 10^{-5} M resveratrol had a doubling time of 60 hr whereas control cells doubled every 30 hr. Trypan blue exclusion assay showed that at concentrations of 10^{-5} M or lower, resveratrol did not affect cell viability (90% viable cells) whereas at 10^{-4} M, only 50% of the cells were viable after 6 days of resveratrol treatment (data not shown). Moreover, MCF-7 cells did not undergo apoptosis after incubation with resveratrol at concentration of 10^{-5} M as determined by ApoAlert Annexin V Apoptosis kit (Clontech) (data not shown).

Effect of resveratrol on the growth of MCF-7 cells cultivated in estrogen-depleted medium

MCF-7 cells were plated in estrogen-depleted PFMEM medium for 24 hr, as described in the Materials and Methods section. Resveratrol was added at the indicated concentrations and cells were counted at day 6 (Fig. 2). Resveratrol alone at a concentration as low as 10^{-7} M had a growth-promoting effect on MCF-7 cells cultivated in estrogen-depleted medium. The stimulatory effect of resveratrol reached a maximum at 10^{-6} M. However, resveratrol was not as potent a growth stimulator as E_2 since the maximal stimulation of MCF-7 cell growth with resveratrol (10^{-6} M) was only twofold over control while a fourfold stimulation was observed with 10^{-9} M E_2 . In contrast, when the cells were cultivated in the estradiol-depleted medium PFMEM, a 10^{-5} M concentration of resveratrol completely inhibited the growth of MCF-7 cells. No evidence of cytotoxicity measured by trypan blue exclusion or apoptosis measured with the ApoAlert Annexin V Apoptosis kit was observed after MCF-7 cells were exposed to resveratrol at concentrations of 10^{-5} M and lower (data not shown).

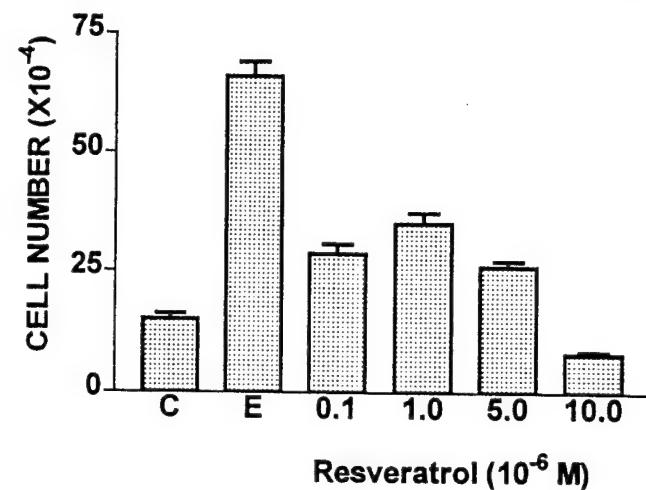


Fig. 2. Effect of resveratrol on the growth of MCF-7 cells in estrogen-depleted medium. MCF-7 cells were plated at 5×10^4 cells per well in 24-well plates in phenol-red free α -MEM plus 5% charcoal-stripped FBS (PFMEM) for 24 hr. The medium was then removed and replaced with the same PFMEM medium in the presence of various concentrations of resveratrol or 10^{-9} M E_2 only ('E') as indicated in the figure. Control cells ('C') received 0.1% ethanol only. The cell number was measured at day 6. Values are mean \pm SD of triplicate determinations.

Resveratrol antagonizes E_2 -mediated stimulation of MCF-7 cell growth

Since E_2 is the major stimulator of MCF-7 cell growth, we next examined the effect of resveratrol on E_2 -mediated MCF-7 cell growth. MCF-7 cells were seeded in PFMEM medium. Indicated concentrations of resveratrol and E_2 were added 24 hr later and cells were counted at day 6. First we examined the effect of 5×10^{-6} M of resveratrol on growth of MCF-7 cells cultivated in the presence of increasing concentrations of E_2 . Figure 3A shows that resveratrol (5×10^{-6} M) dramatically inhibited the growth-promoting effect of E_2 at all concentrations tested (from 10^{-12} to 10^{-9} M). When added in the presence of E_2 up to 10^{-10} M, resveratrol completely inhibited the E_2 -mediated MCF-7 cell growth since the cell number after 6 days was similar to the one measured for control cells maintained in estrogen-depleted medium in the absence of resveratrol.

In the next experiment, MCF-7 cells were cultivated in the presence of a constant E_2 concentration (10^{-9} M) and increasing concentrations of resveratrol in order to examine the antiestrogenic potential of different concentrations of resveratrol. As shown in Figure 3B, resveratrol antagonized the effect of E_2 in a dose-dependent manner, starting from 10^{-6} M. A 50% inhibition of E_2 effect was observed at a resveratrol concentration of 5×10^{-6} M. Maximal growth inhibition was achieved in the presence of 10^{-5} M of resveratrol.

In summary, we show that resveratrol, by itself a weak growth stimulator, antagonizes the growth-stimulatory effect of E_2 in a dose-dependent fashion when added in the presence of E_2 .

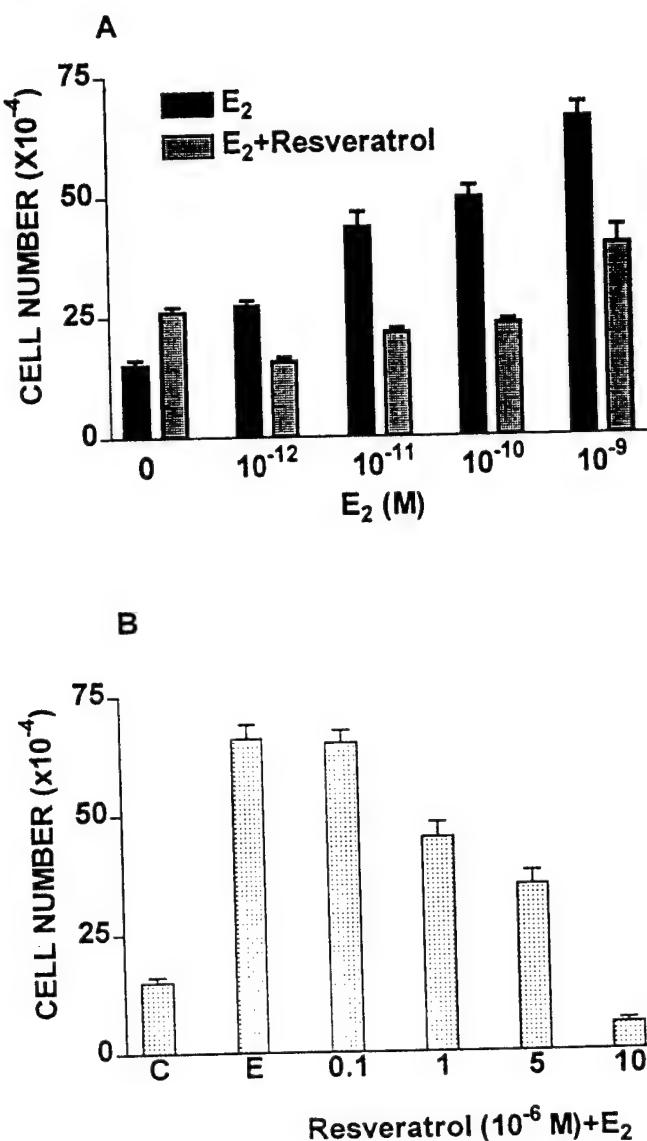


Fig. 3. Resveratrol antagonizes the E_2 -mediated growth in MCF-7 cells. The culture conditions of MCF-7 cells were similar to the ones used in the legend of Figure 2 with indicated concentrations of E_2 and resveratrol. Cells were counted at day 6. Values are mean \pm SD of triplicate determinations. A: Effect of 5×10^{-6} M resveratrol on the MCF-7 cell growth mediated by different concentrations of E_2 . B: Effect of different concentrations of resveratrol on MCF-7 cell growth mediated by 10^{-9} M E_2 . The control cells received either 0.1% ethanol only (C) or 10^{-9} M E_2 only (E).

Resveratrol antagonizes the PR expression stimulated by E_2

In order to investigate further the antiestrogenic action of resveratrol, we examined whether resveratrol antagonized the effect of E_2 on the mRNA expression of an E_2 -inducible gene product such as PR. PR is the most responsive of all the estrogen-regulated RNAs studied so far (May et al., 1989). Using RT-PCR technique, PR mRNA expression was examined in MCF-7 cells treated or not with resveratrol and E_2 . As shown in Figure 4, resveratrol alone stimulated PR expres-

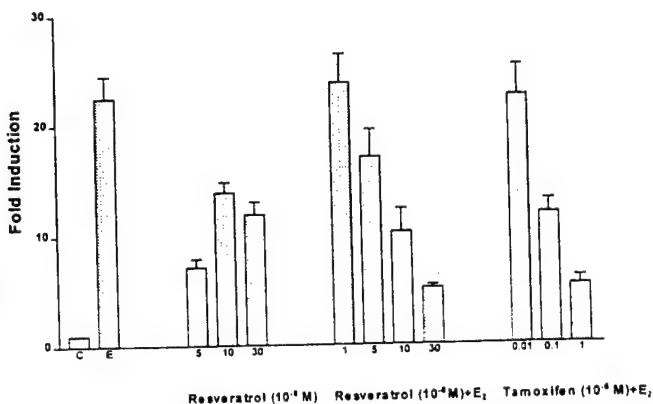


Fig. 4. Effect of resveratrol on PR mRNA expression stimulated by E_2 . MCF-7 cells were plated in DME-F12 medium supplemented with 5% FBS. Two days later, cells were starved in PFMEM medium for 24 hr. Cells were treated either with 0.1% ethanol as control (C); 10^{-9} M E_2 only (E); increasing concentration of resveratrol alone or in the presence of 10^{-9} M E_2 ; or indicated concentrations of 4-OH-tamoxifen in the presence of 10^{-9} M E_2 . Total RNA was isolated 24 hr later. PR mRNA expression was examined using RT-PCR as described in Materials and Methods. The PR signals were scanned and normalized to β -actin internal control and the results were expressed as fold induction in comparison to control. Values correspond to mean \pm SD of three independent experiments.

sion, reaching a maximum of 10-fold at 10^{-5} M ($P < 0.01$) whereas stimulation by E_2 (10^{-9} M) alone was 22-fold ($P < 0.005$). When added in the presence of E_2 (10^{-9} M), resveratrol inhibited PR expression in a dose-dependent manner similarly to the antiestrogen 4-OH-tamoxifen. At 10^{-5} M, resveratrol inhibited PR expression by 50% ($P < 0.002$) and by 75% ($P < 0.02$) at 3×10^{-5} M. The fact that the maximal stimulation of PR mRNA expression obtained with resveratrol alone was only about one half of the one observed with E_2 alone is in agreement with the moderate-growth stimulatory effect of resveratrol on MCF-7 cells cultivated in the absence of E_2 (Fig. 2), suggesting that resveratrol by itself acts as a weak estrogen.

Resveratrol effect on mRNA expressions of autocrine growth factors and their receptors

Cell growth study had shown that long-term incubation of MCF-7 cells with resveratrol in the presence of E_2 dramatically inhibited their growth (80%–90% inhibition after 6 days at 10^{-5} M of resveratrol). Experiments were then performed to investigate the effect of resveratrol on the mRNA expression of growth factors and growth factor receptors synthesized by breast cancer cells and known to be important for their growth. Using RT-PCR, the expression of mRNA for TGF- α , TGF- β s, IGF-I, IGF-II as well as their receptors was examined in MCF-7 cells treated for 6 days with 5×10^{-6} M and 10^{-5} M resveratrol. The most noticeable changes were the significant inhibition of TGF- α and IGF-IR mRNA expressions as well as a dramatic elevation of TGF- β 2 mRNA expression after resveratrol treatment.

As shown in Figure 5, resveratrol inhibited the TGF- α and IGF-IR mRNA expressions in a dose-dependent fashion. 10^{-5} M resveratrol inhibited the expression of TGF- α mRNA by 87% ($P < 0.001$) and

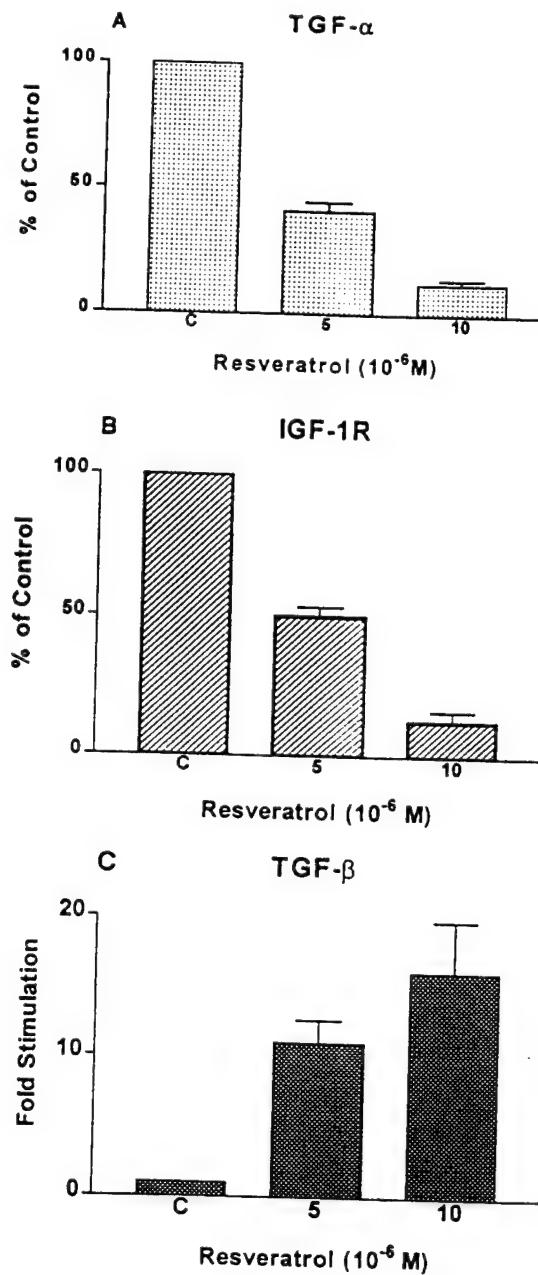


Fig. 5. Effect of resveratrol on the mRNA expressions of TGF- α , IGF-1R, and TGF- β 2. MCF-7 cells were plated in DME-F12 medium plus 5% FBS in the absence of ethanol only, control cells, C) or in the presence of indicated concentrations of resveratrol. Total RNAs were isolated at day 6 and examined for the expressions of TGF- α (A), IGF-1R (B), and TGF- β 2 (C) mRNA by RT-PCR. The signals were normalized to β -actin internal control and the results were expressed as either fold induction or percentage of inhibition in comparison to control. Values are mean \pm SD of three independent experiments.

IGF-1R mRNA by 90% ($P < 0.002$). There was a slight increase in IGF-II mRNA expression while IGF-I mRNA was not detected by RT-PCR (data not shown). No changes were observed in the mRNA expression of IGF-IIR and EGFR after resveratrol treatment (data not shown).

TGF- β 2 has been regarded as a negative growth

regulator for breast cancer cells and a marker for measuring antiestrogenic action in vitro and in vivo since its expression is stimulated by antiestrogen (Butta et al., 1992; Kopp et al., 1995; Knabbe et al., 1996; MacCallum et al., 1996; Muller et al., 1998). A sixfold increase of TGF- β 2 mRNA expression was observed after MCF-7 cells were treated with 5×10^{-6} M resveratrol ($P < 0.01$). At 10^{-5} M, resveratrol significantly stimulated TGF- β 2 mRNA expression up to 15-fold above the control ($P < 0.02$). In contrast to TGF- β 2, no change in the expression of TGF- β 1, TGF- β 3, and TGF- β receptor mRNAs was observed in these conditions (data not shown). Finally, resveratrol (10^{-5} M) had no effect on ER mRNA expression (data not shown). A similar effect of resveratrol on IGF-1R, TGF- β 2, and TGF- α mRNA expression was observed when MCF-7 cells were cultured in PFMEM medium supplemented with E_2 (data not shown).

DISCUSSION

The results presented in this study show that resveratrol inhibits the growth of ER-positive human breast cancer MCF-7 cells. We further demonstrate that resveratrol, itself a partial ER agonist, antagonizes the growth-stimulatory effect of E_2 in a dose-dependent fashion both at the cellular level (cell growth) and at the molecular level (gene activation). At 5×10^{-6} M, resveratrol significantly inhibited MCF-7 cell growth mediated by all concentrations of E_2 tested (10^{-12} M to 10^{-9} M). The antiestrogenic effect of resveratrol could be observed when added at concentrations of 10^{-6} M and above. MCF-7 cells from which resveratrol (10^{-5} M) had been removed after 3 days incubation failed to regain any growth advantage over the cells continuously maintained in the presence of resveratrol (data not shown). Moreover, cells that had been incubated for 3 days with resveratrol (10^{-5} M) and had been replated in medium without resveratrol still continued to grow at a reduced rate (data not shown). These data would suggest that the inhibitory effect of resveratrol on MCF-7 cell growth is irreversible. One possible explanation is that resveratrol may have induced differentiation of the MCF-7 cells. To test this hypothesis, we measured expression of lactalbumin protein, a differentiation marker of mammary cells (Russo et al., 1990). We found that lactalbumin expression measured by immunocytochemistry was inhibited rather than elevated in cells treated for 6 days with 10^{-5} M resveratrol (data not shown). Moreover, we could not detect any change in the mRNA expression of MUC-1 mucin gene, a marker associated with the degree of malignancy of breast cancer (Spicer et al., 1995). Apparently, the irreversible inhibition by resveratrol on the growth of MCF-7 cell was due to reasons other than induction of differentiation.

Resveratrol did not affect the MCF-7 cell viability when used at concentration of 10^{-5} M as determined by trypan blue exclusion assay. However, at 10^{-4} M, only about 50% of the cells treated for 6 days with resveratrol remained viable. Mgbonyebe et al. (1998) observed a significant effect of resveratrol on the viability of breast cancer cells but only at concentrations higher than 5×10^{-5} M. Recently, Clement et al. (1998) also reported that T47D human breast cancer cells underwent apoptosis in the presence of resveratrol ($3.2 \times$

10^{-5} M). We did not observe apoptosis from MCF-7 cells after exposure to resveratrol at concentrations of 10^{-5} M although a significant inhibition of cell growth was observed.

At the molecular level, our data showed that resveratrol antagonized the stimulatory effect of E_2 on PR mRNA expression in a dose-dependent manner. Resveratrol alone activated PR mRNA expression, but when combined with E_2 (10^{-9} M), it inhibited PR mRNA expression. Autocrine growth factors have been shown to play an important role in the regulation of breast cancer cell growth (Yee et al., 1988; Lippman and Dickson, 1989; May and Westley, 1995; Jones and Clemons, 1995; Dickson and Lippman, 1995). Since resveratrol inhibited the growth of MCF-7 cells in the presence of E_2 , it was interesting to examine whether it would affect the expressions of several autocrine growth factors and their receptors. The most obvious changes observed in the cells treated with resveratrol were a significant inhibition of TGF- α mRNA expression (87% inhibition with 10^{-5} M of resveratrol) and of IGF-IR mRNA expression (90% inhibition with 10^{-5} M of resveratrol). TGF- α has a growth-promoting effect on human breast cancer cells in culture. Its expression is induced by E_2 and suppressed by antiestrogen (Lippman et al., 1988; Dickson, 1990). IGF-IR has also been described as a target of estrogen regulation and its induction is important for an E_2 -mediated proliferative effect in MCF-7 cells (Stewart et al., 1990). Since the growth-promoting effect of E_2 requires the presence of TGF- α and IGF-IR, the inhibition of their expression by resveratrol might result in an inhibition of cell growth. Moreover, the fact that resveratrol also inhibited TGF- α and IGF-IR mRNA expression in cells cultivated in PFMEM medium in the presence of E_2 may reflect the antagonism of estrogen action since these two genes are known to be estrogen responsive.

Our results also showed for the first time that resveratrol dramatically stimulated TGF- β 2 mRNA expression (15-fold) in MCF-7 cells without any change in TGF- β 1 and TGF- β 3. TGF- β s have been shown to act as negative autocrine regulators that inhibit the growth of most breast cancer cell lines (Knabbe et al., 1987; Wang 1995, 1996). The TGF- β family includes three isoforms, TGF- β 1, TGF- β 2, and TGF- β 3. Growth stimulation of estrogen-dependent breast cancer cells with E_2 is associated with the down-regulation of TGF- β 2 and TGF- β 3 mRNAs whereas growth inhibition of these cell lines by the antiestrogen (tamoxifen) is associated with elevated TGF- β 2 mRNA expression (Jeng et al., 1993). In fact, part of the growth-inhibitory effect of antiestrogen is thought to be mediated through the induction of TGF- β s (Knabbe et al., 1987; Zugmaier et al., 1989; Arteaga et al., 1990; Arrick et al., 1990; Jeng et al., 1993). It is believed that in MCF-7 cells, antiestrogen compounds first induce the secretion of TGF- β 1 via a nontranscriptional pathway; TGF- β 1 in turn induces TGF- β 2 by a direct transcriptional mechanism (Knabbe et al., 1996). There is still some controversy regarding the role of TGF- β s in the growth-inhibitory effect of antiestrogen. Recently, Koli et al. (1997) reported that blockade of TGF- β signaling using the dominant negative TGF- β 2 receptor in combination with a neutralization assay failed to prevent antiestrogen-mediated growth inhibition of MCF-7

cells. Regardless of the conflicting reports about its importance for the regulation of breast cancer cell growth, TGF- β 2 has been widely accepted as a marker of antiestrogen action in vitro and in vivo (Knabbe et al., 1996; Kopp et al., 1995; MacCallum et al., 1996; Muller et al., 1998). Thus, the stimulation of TGF- β 2 mRNA expression by resveratrol is in agreement with its antiestrogenic effect which is also shown by its ability to inhibit PR mRNA expression stimulated by E_2 . In support of this conclusion, we have shown that resveratrol simulated TGF- β 2 mRNA expression in MCF-7 cells cultivated not only in complete medium but also cultivated in defined medium in the presence of E_2 (data not shown). This also provides a possible mechanism for the effect of resveratrol as a growth inhibitor for MCF-7 cells.

Gehm et al. (1997) have shown by competitive binding of radiolabeled estradiol in the presence of resveratrol with a clone of MCF-7 cells that resveratrol was a weak ligand for the ER. The IC_{50} for resveratrol to inhibit 0.1 nM 125 I-estradiol binding to ER was about 10^{-5} M. They also indicated that resveratrol, acting as a pure ER agonist, stimulated the growth of a clone of MCF-7 cells in the absence of estrogen. However, the effect of resveratrol on cell growth in the presence of E_2 was not examined in this study. While our studies were being carried out, Mgbonyebe et al. (1998) reported that resveratrol was a growth inhibitor of several ER-positive and ER-negative human breast cancer cell lines. However, these studies did not provide any mechanistic explanation for this effect. In our studies, we show that at low concentrations, resveratrol is a partial ER agonist like tamoxifen. However, at higher concentrations, in the presence of E_2 , resveratrol will antagonize the E_2 effect, resulting in growth inhibition of breast cancer cells. Our studies propose that this growth inhibition may be due to the ability of resveratrol to modulate the expression of several autocrine growth modulators and/or their receptors in the breast cancer cells. The most likely mechanism could be an antiestrogenic effect of resveratrol due to direct competition of resveratrol with E_2 for binding to ER as was suggested for other phytochemicals (Adlercreutz et al., 1992; Miksicek, 1993; Ruh et al., 1995). But other mechanisms might also be involved, such as prevention of ER binding to estrogen-responsive element (ERE) or of ER-mediated transactivation. Finally, our data also suggest that the antiestrogenic effect of resveratrol may not be the sole mechanism for its growth-inhibitory effect on breast cancer cells. We have shown that resveratrol is also capable of inhibiting the growth of the ER-negative MDA-MB-468 cells, although less effectively than on the ER-positive MCF-7 cells (Lu and Serrero, unpublished results). Resveratrol has been reported to be a potent inhibitor of ribonucleotide reductase and cyclooxygenase-2 (Fontecave et al., 1998; Subbaramaiah et al., 1998). It can also inhibit NADH:ubiquinone oxidoreductase (Fang and Casida, 1998) and DNA polymerase (Sun et al., 1998). The inhibition of these key enzymes involved in cell metabolism may explain some of the antiproliferative effects of resveratrol, particularly observed in ER-negative cells.

There is no pharmacokinetics information available about resveratrol metabolism in the human body. It is believed that a couple of glasses of wine could provide

a two-digit micromolar concentration of resveratrol where most of the pharmacological effects of resveratrol are observed (Jang et al., 1997). The findings that resveratrol presents antiestrogenic activity and also inhibits the growth of ER-negative breast cancer cells raise an interesting question about its potential role as a chemopreventive agent. People in France have a lower incidence of ischemic heart disease despite their notoriously high fat diet, a phenomenon defined as the French Paradox (Renaud and de Lorgeril, 1993). Consumption of red wine rich in resveratrol is believed to be the most likely cause of this so-called French Paradox (Frankel et al., 1993; Renaud and de Lorgeril, 1993; Soleas et al., 1997). In term of cancer prevention, reports in the literature have also indicated an inverse relationship between breast cancer and wine consumption (not simply alcohol consumption; Renaud and de Lorgeril, 1993; Gronbaek et al., 1995). Based on this report and the data presented here, resveratrol is definitely an interesting compound worthy of further investigation for its chemopreventive potential.

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Inhibition of tumorigenicity of estrogen receptor negative human breast carcinoma MDA-MB-468 cells by transfection of antisense cDNA for PC-Cell Derived Growth Factor (PCDG; granulin precursor).

Runqing Lu¹, and Ginette Serrero^{1,2*}

1 Department of Pharmaceutical Sciences

University of Maryland School of Pharmacy

2 Program of Oncology

University of Maryland Marlene and Stewart Greenebaum Cancer Center

20 N. Pine Street

Baltimore, MD 21201, USA

Footnotes

* To whom correspondence should be addressed. Tel.: 410-706-6639;

Fax: 410-706-0346; Email: gserrero@rx.umaryland.edu

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2- The Abbreviations used are: DME, Dulbecco' Modified Eagle's medium; E₂, 17-β estradiol; ER, estrogen receptor; ERE, estrogen responsive element; FBS, fetal bovine serum; IGF-I, II, Insulin like Growth Factor I, II; IGF-IR, insulin like growth factor I receptor; α-MEM, α-modified Eagle's medium; MBP, myelin basic protein; PCDGF, PC cell derived growth factor; PFMEM, phenol-red free α-MEM plus 5% charcoal-stripped FBS; TGF-α, transforming growth factor α;

3- You, J. and Serrero, G. manuscript in preparation

ABSTRACT

PC-cell derived growth factor (PCDGF) is an 88 kDa glycoprotein originally purified from the conditioned medium of the highly tumorigenic mesenchymal teratoma-derived cell line PC and corresponding to the precursor of the 6 kDa polypeptides named granulins or epithelins.

PCDGF has been shown to be overexpressed in the highly tumorigenic PC cell line and to stimulate its growth in an autocrine fashion. We have demonstrated that PCDGF overexpression in the teratoma-derived cell and its overexpression is essential for the cell tumorigenicity. We recently reported that PCDGF was expressed in several estrogen receptor positive (ER⁺) human mammary epithelial cells and stimulated by estrogen in a dose- and time-dependent fashion. In the present paper, we investigated PCDGF mRNA and protein expression in a variety of estrogen receptor positive (ER⁺) and estrogen receptor negative (ER⁻) human breast cancer cell lines. Noryhern blot and western blot analysis indicate that the levels of PCDGF mRNA and protein expression were low in non-tumorigenic, immortalized human mammary epithelial cells and increased in human breast carcinomas in a positive correlation with the degree of tumorigenicity. Treatment of the ER⁻ MDA-MB-468 cells with anti-human PCDGF neutralizing antibody resulted in a dose-dependent inhibition of their proliferation suggesting that PCDGF acts as an autocrine growth factor for these cells. Experiments were performed to determine whether the autocrine production of PCDGF was involved in the tumorigenicity of the cells. For this purpose, we examined the in vitro and in vivo growth properties of MDA-MB-468 cells where PCDGF expression had been inhibited by stable transfection of antisense PCDGF cDNA. The results show that inhibition of PCDGF expression resulted in a reduced cell proliferation rate *in vitro*. In